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Polysubstrate Monooxygenases and Other Xenobiotic-Metabolizing Enzymes in Susceptible and Resistant Colorado Potato Beetle

KABEER I. AHAMMAD-SAHIB, ROBERT M. HOLLINGWORTH, MARK E. WHALON, PHILIPPOS M. IOANNIDIS, AND EDWARD J. GRAFIUS

Pesticide Research Center and Department of Entomology, Michigan State University, East Lansing, Michigan 48824

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The activities of key defensive enzymes against xenobiotics were studied in two azinphosmethyl-resistant and one susceptible strain of Colorado potato beetle (*Lepinotarsa decemlineata* Say) with emphasis on the microsomal polysubstrate monooxygenase system. Monooxygenase activity comparable to that in other coleopterans was observed in the oxidation of NADPH, O-demethylation of *p*-nitroanisole, N-demethylation of aminopyrine, and epoxidation of aldrin. Aniline hydroxylase activity was not detected. Monooxygenase activity against aminopyrine was largely confined to the microsomal fraction of homogenates and was more active in the larval gut than in the fat body. Microsomal NADPH oxidation was inhibited strongly *in vitro* by SKF 525-A and *in vivo* by prior treatment of insects with piperonyl butoxide. The activity of the monooxygenase system was approximately twice as high in the resistant strains as that in the susceptible strain. Glutathione transferase activity was also elevated in the resistant strains, but no elevation in carboxyesterase activity was seen. Piperonyl butoxide pretreatment increased the toxicity of azinphosmethyl several-fold more strongly in the resistant than susceptible strain, indicating a significant role for monooxygenase activity in resistance. However, in both strains, resistance to azinphosmethyl is multifactorial and enhanced monooxygenase activity is only one contributing factor. © 1994 Academic Press, Inc.

INTRODUCTION

The Colorado potato beetle (CPB)[1] *Leptinotarsa decemlineata* (Say), is a serious pest of potato, tomato, and egg plant in North America and elsewhere. Despite considerable research on biological controls for this pest, success has been limited and potato growers continue to depend heavily on insecticides. The consequent development of resistance in CPB to organochlorine, organophosphorus, carbamate, and pyrethroid insecticides has been remarkably rapid and is now widespread. The CPB had developed resistance to all the major classes of modern synthetic insecticides on Long Island, New York by 1983 (1). Subsequently, CPB resistance has been detected in the midwest (2) and western United States (3). Control failures involving all major insecticides are increasingly common in the midwest (4). Both in the northeast and midwest, resistance to such compounds as fenvalerate, azinphosmethyl, and rotenone has been temporarily overcome by the incorporation of the synergist, piperonyl butoxide (PBO), as an inhibitor of resistance-inducing polysubstrate monooxygenases (PSMOS) (1, 5). Many growers now face highly resistant CPB populations with only marginally effective insecticides remaining and unknown prospects of new compounds (5). There is a critical need for resistance management programs for the CPB.

Insecticide resistance management is predicated on the ability to detect resistance at very low allele frequencies in the treated population so that action can be

[1] Abbreviations used: AChE, acetylcholinesterase; APM, azinphosmethyl; CPB, Colorado potato beetle; CDNB, 1-chloro-2,4-dinitrobenzene; DEF, *S,S,S,*-tributyl phosphorotrithioate; DEM, diethyl maleate; PSMO, polysubstrate monooxygenase; PBO, piperonyl butoxide; RLI, Long Island resistant strain; RM, JP-Macomb resistant strain; S, Arizona susceptible strain; SR, synergistic ratio.

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taken before insects become homozygous in this trait. Recent advances in biochemical and molecular biological techniques for detecting resistance in management programs were reviewed by Brown and Brogdon (6). They point out that the ability to detect resistance depends on understanding the basic resistance mechanism, and identification of the mechanism is essential for the intelligent choice of replacement compounds and the avoidance of cross-resistance. As a preliminary step in instituting resistance management programs for CPB, field screening methods have been developed to monitor resistance types and levels (4).

The study of pesticide resistance mechanisms in a variety of insects reveals that there are three major physiological paths to resistance: decreased uptake, increased detoxification, and changed target sites (7). Unfortunately, knowledge of resistance mechanisms in CPB is slight. In this study, our objectives were to (i) conduct synergism studies to indicate mechanisms of resistance to azinphosmethyl in two resistant CPB strains, and (ii) evaluate the enzyme system involved by utilizing *in vitro* assays. In addition to the highly resistant Long Island strain, we have also studied the metabolic resistance mechanisms in a moderate to highly resistant field-collected strain from Macomb County, Michigan. By this comparative analysis we can begin to understand the basic resistance mechanisms as needed to develop effective resistance management strategies for current and future CPB insecticides.

MATERIALS AND METHODS

Chemicals. 5Azinphosmethyl (APM; technical grade, ≥90% pure) was obtained from Mobay Chemical Corp. (Kansas City, MO). The synergist, piperonyl butoxide (PBO, 99% pure), was a gift from McLaughlin, Gormley, King Co. (Minneapolis, MN). *S,S,S,*-Tributyl phosphorotrithioate (DEF, 95% pure) was a gift from F. Matsumura (Pesticide Research Center, Michigan State University, East Lansing, MI). SKF 525-A was obtained from Smith, Kline, and French Laboratories (Philadelphia, PA). All other biochemicals and chemicals were obtained either from Sigma Chemical Co. (St. Louis, MO) or from Aldrich Chemical Co. (Milwaukee, WI).

Insect strains. CPB eggs were transferred to young potato plants at a rate of approximately 2–3 egg masses per plant. As the larvae developed, they were transferred to fresh plants. Adult beetles (six of each sex) were placed in mating and oviposition cages (1 m^3) containing plants. Oviposition cages were sampled daily for eggs which were removed and handled as above. The greenhouse was maintained at 26 ± 2°C and a 16-L:8-D photoperiod.

The susceptible (Arizona) strain was originally collected in an isolated area in Arizona from silver-leaf night shade, *Solanum nigrum,* and has been maintained here since 1986 without exposure to insecticides. It shows high sensitivity to all insecticides so far examined. The Long Island-APM resistant strain (RLI) was originally collected from a potato field in Long Island, New York and was provided by Dr. A. J. Forgash (Rutgers University, N.J.). This strain had been selected further with APM (80% mortality) for six generations in the laboratory at the time of this study. During selection, resistance to APM was enhanced, but the original resistance to permethrin was greatly decreased. High resistance to carbofuran was maintained. The other resistant strain (RM) was collected from a potato field in Macomb County, Michigan. This strain was not selected with any insecticide in the laboratory, but was over 100-fold resistant to APM, permethrin, and carbofuran.

Biological assay. The dose–response test and the standard bioassay techniques for CPB were as proposed by FAO (8). Synergists [PBO, DEF, and diethyl maleate (DEM)] were applied 2 hr prior to the insecticide treatment at the ratio of 1:4 insecticide:synergist, respectively. Both insecti-

cide and synergist treatments were topical application of 2-μl aliquots in acetone to the abdominal sternites. Sixteen adults, 6–10 days old, of mixed sexes were used at each dose. Beetles were held at 26 ± 2°C for 3 days before assessing mortality. Each bioassay was based on four to six insecticide concentrations selected after preliminary assays to give mortalities in the range 10–90%. Acetone-treated or synergist-treated beetles were used as controls. Generally, control mortality was less than 10%. Tests with greater than 20% control mortality were rejected. Data from at least two bioassays were combined and analyzed by probit analysis using the SAS program (9).

To estimate *in vivo* effects of synergists on microsomal oxidative activity, adult beetles were treated with 100 μg/beetle of synergists 2 hr before killing by dipping them into liquid nitrogen. Microsomes were prepared from the abdomens as described below.

Isolation of microsomes. Microsomes were generally prepared (10) from the bodies of adult CPB after removing the head. The tissue was homogenized in potassium phosphate buffer (0.2 *M*, pH 7.2) in a motor-driven glass homogenizer with a Teflon pestle. These homogenates were prepared to contain five or six bodies per milliliter. The initial homogenates were centrifuged at 12,000*g* for 15 min and the postmitochondrial supernatant was centrifuged for 1 hr at 105,000*g* to sediment the microsomal fraction.

To compare the activity of PSMOs between larvae and adults, the tissues of 20 fourth-instar larvae of strain RLI were assayed for PSMO activity. One-third of the larvae were used for preparing microsomes from the whole abdomen. The rest of the larvae were dissected and fat bodies and guts were isolated. Gut contents were removed and the lumen rinsed with buffered saline. The microsomes were isolated from the three larval tissue as before.

For experiments on O-demethylation, N-demethylation, and hydroxylation the microsomal pellet was resuspended in phosphate buffer (0.2 *M*, pH 7.2) containing 2% bovine serum albumin. For the determination of NADPH oxidation the pellet was resuspended in three times the original volume of the same phosphate buffer and was resedimented at 105,000*g*, and the second pellet was resuspended in the phosphate buffer. This procedure was included to remove any soluble diaphorase which can interfere with microsomal NADPH oxidase measurements.

Microsomal oxidations. The following reactions associated with microsomal PSMOs were assayed as described below.

NADPH oxidation. Activity was determined by the decrease of absorbance at 340 nm in a double beam spectrophotometer as described by Folsom and Hodgson (10). The reference cell contained all reactants except NADPH. The amount of NADPH oxidized was determined from its extinction coefficient (e_{340} = 6.22 mM^{-1} cm^{-1}). The microsomal suspension containing 50–500 μg protein was made to 2.5 ml with phosphate buffer followed by 0.5 ml NADPH (0.3 μmol). To characterize this activity further, the sensitivity to SKF 525-A, an PSMO inhibitor, was determined at two concentrations (0.2 and 2.0 m*M*).

NADPH: Cytochrome c reductase. This activity was measured by following the reduction of cytochrome c at 550 nm at 26°C (11). The activity was calculated using the extinction coefficient of 21.1 mM^{-1} cm^{-1}. The total reaction mixture in a final volume of 2.5 ml contained 70–100 μg microsomal protein, 0.02 m*M* cytochrome c, and 0.1 m*M* NADPH in 0.05 *M* phosphate buffer, pH 7.5.

p-Nitroanisole O-demethylase. This activity was estimated by measuring the production of *p*-nitrophenol spectrophotometrically according to Kinoshita *et al.* (12). *p*-Nitroanisole was dissolved in ethanol: propylene glycol (1:4) at 16 mg/per milliliter and then diluted 10-fold with 0.1 *M*, pH 7.8, phosphate buffer. The reaction mixture contained 2.9 m*M* *p*-nitroanisole, 3.3 m*M*

NADPH, and 50–100 μg microsomal protein in 0.7 ml 60 m*M* phosphate buffer, pH 7.8. The reactions were incubated at 37°C for 60 min in a metabolic shaker and stopped by adding 2.5 ml cold acetone plus 0.2 ml of glycine–NaOH buffer (0.5 *M*, pH 9.5). After centrifugation, the optical density of the clear supernatant was read at 410 nm. Enzyme activity was calculated using a *p*-nitrophenol standard.

Aminopyrine N-demethylase. Activity was measured by a method based on that of LaDu *et al.* (13). Aminopyrine was initially dissolved in ethanol:propylene glycol (1:4) as a 130-m*M* stock solution. In a final volume of 1.6 ml, the reaction mixture contained 8 m*M* aminopyrine, 1.4 m*M* NADPH, 12.5 m*M* semicarbazide, 170–230 μg microsomal protein, and 150 m*M* phosphate buffer at pH 7.5. After incubation for 60 min at 37°C, the reaction was stopped by the addition of 1 ml 20% TCA. The precipitate was removed by centrifugation and to 1 ml of the clear supernatant was added 1 ml Nash reagent (2 *M* ammonium acetate, 0.05 *M* glacial acetic acid, and 0.02 *M* acetylacetone). The tubes were incubated at 37°C for 40 min and the color due to the presence of formaldehyde was read at 412 nm. The enzyme activity was calculated using a 37% formaldehyde standard (its w/v ratio was utilized for calculating molarity of the standard).

Aniline hydroxylase. The formation of *p*-aminophenol from aniline was estimated according to the method of Brodie and Axelrod (14). The reaction mixture consisted of 150 m*M* phosphate buffer, 5 m*M* aniline hydrochloride, 4.1 m*M* NADPH, and 50–150 μg microsomal protein in a final volume of 2 ml, pH 7.5. After incubation for 30 min at 37°C, the reaction was stopped by adding 1 ml of 10% TCA. Following centrifugation, 0.5 ml 20% Na_2CO_3 and 1 ml 2% phenol in 0.2 *N* NaOH was added to 1 ml of supernatant. The absorbance was measured at 630 nm after 30 min at room temperature. A *p*-aminophenol standard curve was used to quantify the enzyme activity.

Aldrin epoxidase. The method used was that of Yu *et al.* (15). The 5-ml reaction mixture contained 150–200 μg microsomal protein, 75 m*M* phosphate buffer, pH 7.5, an NADPH generating system consisting of 1.8 μmol NADP, 18 μmol glucose-6-phosphate, and 1 unit of glucose-6-phosphate dehydrogenase and 250 nmol aldrin added in 0.1 ml methylcellosolve. Duplicate assays were conducted with shaking at 40°C for 5 min. The reaction product, dieldrin, was extracted with 10 ml hexane and analyzed by GLC (Perkin–Elmer 8500) using electron capture detection (15).

Acetylcholinesterase (AChE). This esterase was assayed by the method of Ellman *et al.* (16). The reaction mixture (3.2 ml) contained 0.1 *M* sodium phosphate buffer, pH 8.0, 0.63 m*M* dithiobisnitrobenzoic acid, and 75–150 μg microsomal protein. The reaction was initiated by adding acetylthiocholine iodide (0.5 m*M* final concentration) and the increase in absorbance at 412 nm was monitored. The enzyme activity was calculated using extinction coefficient of 13.6 mM^{-1} cm^{-1} (16).

Carboxylesterase activity. The method of van Asperen (17) was used. In a 5-ml final volume were mixed 0.2 m*M* α-naphthyl acetate in 30 m*M* sodium phosphate buffer containing 1% acetone, 10 μ*M* eserine sulfate, 10 μ*M* *p*-(hydroxymercuri) benzoate, and 70–130 μg microsomal protein. After incubation at room temperature for 30 min, 1 ml of a mixture of diazoblue B (0.3%) and SDS (3.6%) was added. After centrifugation, the developed color was measured at 600 nm. The enzyme activity was calculated using an α-naphthol standard curve.

Glutathione transferase. Activity was measured with 1-chloro-2,4-dinitrobenzene (CDNB) according to Habig *et al.* (18). In a final volume of 3.0 ml the reaction mixture contained 5 m*M* GSH, 0.1 *M* potassium phosphate, pH 6.5, microsomal (105,000*g*) supernatant fraction containing 150 to 500 μg protein, and 1 m*M* CDNB. After adding CDNB, the reaction was followed at 340 nm for 3 to 4 min and activity was calculated using the extinction coefficient of 9.6 mM^{-1} cm^{-1}.

Cytochrome P450. P450 was assayed by the method of Omura and Sato (19). P450 content was calculated by using the extinction coefficient of 91 mM^{-1} cm^{-1}. P420 content was calculated using an extinction coefficient of 110 mM^{-1} cm^{-1} as described by Guengerich (20).

Protein. This was determined according to the method of Lowry *et al.* (21) using bovine serum albumin as the standard.

Data analysis. For each assay, determinations were made in duplicate and two to five replicate assays were used to determine the mean reaction rate. Significance ($P = 0.05$) was calculated using a student *t* test.

RESULTS

Resistance and synergistic ratios. As shown in Table 1, the LD_{50} value obtained for the S strain with APM was 1.46 μg/beetle. The RLI and RM strains were much less sensitive with LD_{50} values of 556 and 272 μg/beetle, respectively. The RLI strain thus showed a resistance ratio (R/S) of 381 compared to 186 for the Macomb strain. Slopes of the log dosage–probit lines were much steeper for the RLI than for the RM strain, reflecting the homogeneity of RLI after repeated selection with APM and the heterogeneity in the field-collected RM insects.

Three synergists were selected to help identify the possible mechanisms underlying the insensitivity of the two resistant populations: piperonyl butoxide, a potent inhibitor of PSMOs; DEF, an inhibitor of carboxylesterases; and DEM, an inhibitor of GSH conjugation. The ratio between the LD_{50} values without and with synergist was termed the synergistic ratio (SR). The LD_{50} value of APM to the RLI population decreased from 556 to 27.6 μg/beetle after pretreatment with PBO, exhibiting an SR of 20.1 (Table 1). In the RM strain, PBO had an SR of 16.5. The effect of DEF was lower. It decreased the LD_{50} value for the RLI population to 63.1 μg (SR = 8.8). DEM had little effect on the LD_{50} values since the SR was only 1.9 for the RLI population.

Microsomal NADPH oxidation. Microsomal oxidation of NADPH was linear with time up to 20 min with R/S rate ratios ranging from 1.2 to 1.9 for RM and 2.1 to 2.9 for the RLI population (Fig. 1A).

Since the oxidation of NADPH in these assays could be attributable to reactions other than cytochrome P450 oxidations, the effect of SKF 525-A, a known inhibitor of cytochrome P450, was studied to learn if the oxidation was indeed due to cytochrome P450. The inhibition caused by 0.2 and 2.0 m*M* SKF 525-A was found to be 46.4 and 94% in S, 30 and 84% in RLI, and

TABLE 1

Evaluations of Resistance and Synergism with Azinphosmethyl in Adults from Three Strains of Colorado Potato Beetle

Strain	Synergist[a]	LD_{50} (95% C.I.) (μg/beetle)	Slope[b]	Synergistic ratio
Arizona (S)	None	1.46 (1.05–2.03)	2.6	—
	PBO	0.32 (0.24–0.42)	3.8	4.6
Long Island (RLI)	None	556 (402–770)	3.4	—
	PBO	27.6 (20.2–36.9)	1.9	20.1
	DEF	63.1 (39.9–99.7)	1.4	8.8
	DEM	289 (132–617)	1.4	1.9
Macomb (RM)	None	272 (65–146)	0.79	—
	PBO	16.5 (9.4–25.3)	3.4	16.5
	DEM	36.0 (25.7–51.4)	3.2	7.6

[a] Azinphosmethyl:Synergist ratio = 1:4.

[b] Slope of the log dose–probit % mortality plot.

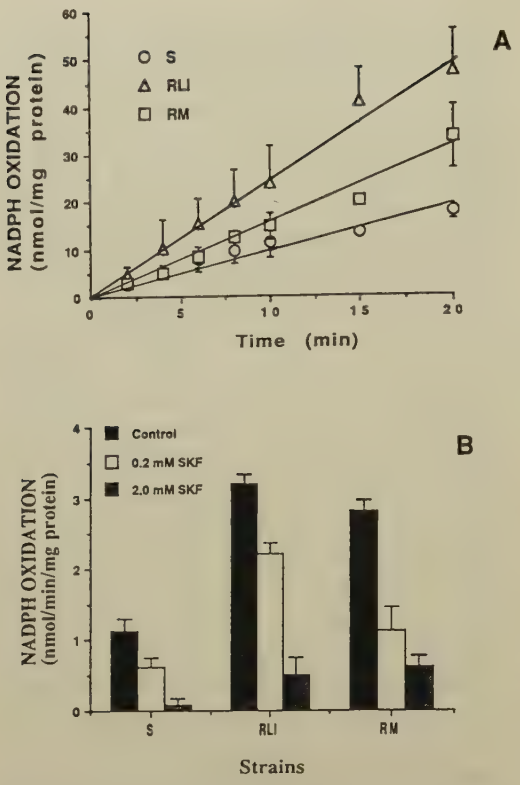

FIG. 1. *(A) Oxidation of NADPH by microsomes from Arizona (S), JP Macomb (RM), and Long Island (RLI) strains of Colorado potato beetle. (B) Effect of the microsomal monooxygenase inhibitor SKF 252-A on endogenous NADPH oxidation by microsomes from susceptible (S) and resistant (RM, RLI) strains of Colorado potato beetle. Data are means ± SD (n = 3 to 4).*

60 and 78% in the RM strains, respectively. (Fig. 1B).

NADPH–cytochrome c reductase activity. The activity of the flavoprotein enzyme which transfers electrons from NADPH to cytochrome P450 was studied in the three strains (Fig. 2). The activity in the S and in both R populations was linear with time up to 10 min. For the duration of time studied, the R/S ratios for rates of reduction ranged from 2.2 to 2.5 for RLI and 6 to 7.8 for RM strains.

Effects of synergist treatment in vivo on MFO activity. The two resistant strains were treated with 100 μg/beetle of PBO, DEF, or DEM for 2 hr and the levels of

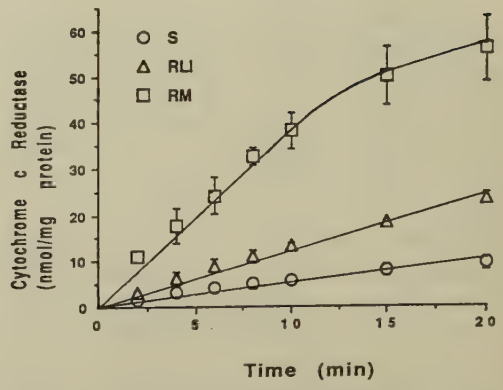

FIG. 2. *NADPH–cytochrome c reductase activity in a microsomal preparation from susceptible (S) and two resistant (RM, RLI) strains of Colorado potato beetle. Data are means ± SD (n = 3).*

microsomal NADPH oxidation and cytochrome c reductase activity were then determined (Table 2). After PBO treatment, the level of NADPH oxidation showed a strong (76 and 79%) decrease in the RLI and RM strains, respectively. DEF treatment caused a 21% decrease in oxidation by RLI microsomes and a 10% decrease in the RM strain, but neither decrease was statistically significant. DEM had no significant effect on microsomal NADPH oxidation. None of the synergists exerted any significant effect on the cytochrome c reductase activity.

Comparative oxidation of xenobiotics. The ability of microsomes from all three strains to carry out four different types of oxidation was assessed using xenobiotic substrates (Table 3). No ring oxidation of aniline could be detected in microsomes from the S or RLI strains (RM strain was not studied). However, the O-demethylation of *p*-nitroanisole, N-demethylation of aminopyrine, and epoxidation of aldrin were all readily measurable. The highest reaction rate was observed with aminopyrine. The reaction rates were similar in microsomes from the two R strains and these were invariably higher than those in the S strain. The ratio of rates (R/S) varied from 1.44 for the RLI strain with *p*-nitroanisole to 2.42 for this strain with aldrin epoxidation.

TABLE 2

Effects of Synergist Pretreatments in Vivo on the Levels of Microsomal NADPH Oxidation and Cytochrome-c Reductase Activity in Long Island (RLI) and JP McComb (RM) Resistant Strains of Colorado Potato Beetle

Experimental condition	NADPH oxidation[a] (nmol/mg protein/min)	Cytochrome c reductase[a] (nmol/mg protein/min)
	RLI	
Control	4.51 ± 0.58	4.80 ± 0.50
+PBO	1.08 ± 0.21[b]	4.44 ± 0.33
+DEF	3.58 ± 0.23	5.36 ± 0.32
+DEM	3.79 ± 0.11	4.85 ± 0.78
	RM	
Control	2.14 ± 0.18	5.23 ± 0.42
+PBO	0.46 ± 0.15[b]	4.71 ± 0.62
+DEF	1.93 ± 0.11	4.62 ± 0.48
+DEM	2.31 ± 0.29	5.14 ± 0.31

[a] Results are mean ± SD of three or more independent assays. Each assay consisted of 10–12 CPB.

[b] Values significantly different ($P < 0.05$) from corresponding controls.

Aminopyrene *N*-demethylase activity was also measured in microsomes from the whole abdomen, the gut, and the fat body from fourth-instar larvae of the RLI strain (Table 4). Specific activity in the whole abdomen microsomes was slightly higher than that in those from the fat body. The gut microsomes showed an approximately three-fold higher specific activity than those from the fat body, but both tissues displayed significant activity.

Also, using aminopyrene, the subcellular distribution of oxidase activity was determined in homogenates of the bodies of adult beetles of the RLI strain (Table 5). The highest specific activity was found in the microsomal fraction with virtually no activity in the mitochondria or the microsomal supernatant. The P450 and P420 content of the microsomal fraction was 0.12–0.15 and 0.07–0.10 nmol per milligram protein, respectively. Microsomes resuspended

TABLE 3

Activity of Selected Monooxygenase Reactions in the Body Microsomes of Adult Arizona (S), Long Island (RLI) and JP McComb (RM) Strains of Colorado Potato Beetle

Reaction	Enzyme activity[a]				
	S	RLI	RLI/S	RM	RM/S
p-Nitroanisole *O*-demethylase	17.5 (±2.04)	25.0 (±2.23)*	1.44	25.9 (±0.25)*	1.47
Aminopyrine *N*-demethylase	49.5 (±4.78)	104.0 (±23.30)*	2.10	81.6 (±17.4)*	1.65
Aldrin epoxidase	0.50 (±0.04)	1.20 (±0.08)*	2.42	1.14 (±0.007)*	2.28
Aniline hydroxylase	ND[b]	ND[b]	—	NS[c]	—

[a] nmol/mg protein/hr for demethylases, nmol/mg protein/5 min for epoxidase. Values are mean ± SD of three to five independent assays. Each assay consisted of tissue from 10 to 12 beetles.

[b] ND, not detectable.

[c] NS, not studied.

* Values significantly different from S activity ($P < 0.05$).

TABLE 4
Aminopyrene N-demethylase Activity of Microsomes from Whole Abdomen, Gut, and Body of Long Island (RLI) Colorado Potato Beetle Larvae

Tissue	Aminopyrene *N*-demethylase[a] (nmol/mg protein/h)	Relative specific activity
Abdomen	145 ± 15	1.00
Gut	340 ± 34*	2.34
Fat Body	110 ± 36	0.76

[a] Results are mean ± SD of three independent assays. Each assay consisted of four to six larvae.

* Significantly different ($P < 0.05$) from abdomen value.

in 20% glycerol showed higher ratios of P450:P420 (typically less than 10% P420; Fig. 3) but, for unknown reasons, they were catalytically less active than those in a glycerol-free medium, and were not used further in this study. NADPH was more effective than NADH in stimulating oxidation in the microsomal fraction. Only in the mitochondrial fraction did NADH prove effective as a source of reducing equivalents and the rate in this case was very low. The combination of NADH and NADPH was generally less effective than NADPH alone. The yield of PSMO activity declined regularly during the fractionation, i.e., dropping from 32 nmol/min/20 insects in the 700*g* supernatant to 23 nmol in the 13,000*g* supernatant to 12 nmol in the microsomal fraction. No measurable *N*-demethylase activity is being removed in the mitochondrial fraction or 100,000*g* supernatant to explain these losses. If all the activity in the whole homogenate is due to the microsomes, the data in Table 5 indicate that only 38% of the initial activity is being recovered in the microsomal fraction.

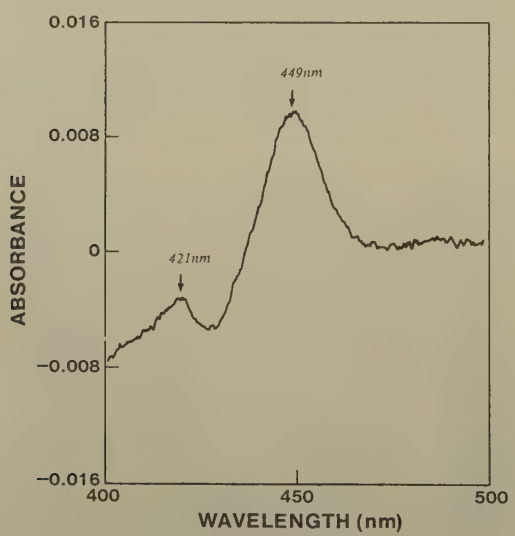

FIG. 3. *Reduced carbon monoxide difference spectrum of microsomes from the RLI strain showing cytochrome absorption peaks at approximately 421 and 449 nM. Microsomes were resuspended in 0.1 mM phosphate buffer (pH 7.4) containing 20% glycerol, 0.4 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol, and 0.1 mM EDTA.*

Other enzymes potentially involved in resistance. To assess the possible involvement of other insecticide resistance mechanisms in the RLI and RM strains, the activity of AChE, carboxylesterases, and glutathione transferase was studied (Table 6). The activity of AChE in the S strain was 8.5 nmol/mg protein/min. In the RLI strain the AChE activity showed no difference from that in the S strain, but in the RM strain the AChE activity was 45% greater than that in the S strain. Only minor increases were seen in carboxylesterase activity in the two R strains, but the activity levels of glutathione transferase in RLI and RM strains were 119 and 87% greater than those in the S strain.

DISCUSSION

Both the APM-selected RLI strain and the field-collected RM strain showed a high level of resistance to APM (381- and 186-fold, respectively) compared to the S strain. With both resistant strains, pretreatment with PBO greatly reduced APM resistance and to a similar degree (16- to 20-fold), although not to the level of susceptibility of the S strain. Pretreatment of the S strain with PBO also resulted in synergism but to a lesser extent (4.6-fold). Since PBO is a relatively specific and potent inhibitor of the PSMO system (22), this provides strong evidence that PSMOs are a significant factor underlying resistance to APM in both strains.

The carboxylesterase inhibitor, DEF,

TABLE 5

Yield and Specific Activity of Aminopyrene N-demethylase Activity in Subcellular Fractions from the Abdomen of Adults of the RLI Strain of Colorado Potato Beetle

Fraction	Yield[a]		Specific activity (nmol/mg protein/min)		
	Activity[b]	Protein[c]	NADPH	NADH	NADPH + NADH
Whole Homogenate	2.18 ± 0.38	4.33	0.51 ± 0.09	0.35 ± 0.08	0.48 ± 0.03
700*g* Supernatant	1.62 ± 0.33	2.53	0.64 ± 0.42	0.33 ± 0.08	0.58 ± 0.03
Mitochondria (13,000*g* pellet)	0.01 ± 0.02	0.54	ND[d]	0.16 ± 0.04	ND
13,000*g* Supernatant	1.17 ± 0.03	1.98	0.61 ± 0.04	0.70 ± 0.08	0.78 ± 0.08
Microsomes (100,000*g* pellet)	0.61 ± 0.16	0.43	1.45 ± 0.37	0.61 ± 0.02	0.73 ± 0.09
Soluble fraction	0.07 ± 0.10	1.06	ND	ND	ND

[a] Each value is the mean ± SE of two individual assays with 20 beetles/assay.
[b] NADPH-dependant; nmol/min/insect.
[c] mg/insect.
[d] ND, not detectable.

showed a clear but lower degree of synergism with the R strains (seven- to ninefold). This could indicate a role for elevated carboxyesterase activity in resistance to APM, but the two resistant strains did not have a notably elevated level of esterase activity against α-naphthylacetate when assayed *in vitro*. It is also possible that DEF inhibits PSMO activity, as observed by Bull and Whitten (23), in the microsomal metabolism of aldrin, but microsomal preparations from beetles treated with DEF showed only a small reduction in ability to oxidize NADPH, a reaction that is strongly inhibited in insects exposed to PBO. Additionally, DEF may be acting like another carboxyesterase inhibitor, tri-*o*-tolylphosphate, in mammals (24) to block nonspecific phosphorylation sites and thus reduce nontarget binding of APM and its oxon.

Although the R strains have a higher GSH transferase activity against CDNB than the S strain, and APM is a probable substrate for this transferase action (25), the lack of synergism by DEM suggests that this group of enzymes is not an important factor in resistance to APM. The elevated GSH transferase activity accompanies higher PSMO activity in both of the R strains. This is a common finding in resistant insects. Plapp (26) notes that in the house fly, elevated GSH transferase activity is never found in the absence of other

TABLE 6

Activity of Acetylcholinesterase, Carboxylesterase, and Glutathione Transferase in Tissue Subfractions from Susceptible and Resistant Strains of Colorado Potato Beetle

Enzyme[a]	Strain		
	S	RLI	RM
Acetylcholinesterase[b]	8.5 ± 2.0	7.7 ± 2.5	12.3 ± 1.3[d]
Carboxylesterase[b]	8.5 ± 1.2	10.2 ± 0.5	12.3 ± 0.1[d]
Glutathione transferase[c]	113 ± 9	248 ± 1[d]	212 ± 7[d]

[a] Values are mean ± SD of three to five independent assays. Each assay consisted of tissue from 10 to 12 insects.
[b] nmol/mg protein/min; microsomal fraction.
[c] nmol/mg protein/min; 105,000*g* supernatant.
[d] Significantly different from S strain ($P < 0.05$).

increased metabolic defenses and that the genes for both PSMO and transferase activity are located on the same chromosome. Some type of linkage or coordinated selection of multiple xenobiotic metabolic systems (26) may also be operating in the resistant CPBs.

Our results with synergists differ somewhat from those of a comparable study by Argentine *et al.* (27). Synergistic ratios for DEF and DEM with APM were very similar to those reported here, but the effect of PBO was much lower (SR = 4, compared to 20 in this study). This may be due to biochemical differences between the strains used, but it is also worth noting that Argentine *et al.* (27) used much lower amounts of PBO (5 μg/insect) than in the present study (up to 100 μg/insect). In the only previous study of PSMO activity in this beetle, Rose and Brindley (28) found a small (10–20%) increase in NADPH oxidation in microsomes from carbamate-resistant insects compared to those from a susceptible strain, but no O-demethylation of *p*-nitroanisole or other xenobiotic oxidations could be detected. In this study, we could not detect aniline hydroxylase activity, but several other standard PSMO reactions, such as aldrin epoxidation, *p*-nitroanisole O-demethylation, and aminopyrine N-demethylation, were readily detectable.

The rates of aldrin epoxidation in the three CPB strains (100 to 240 pmol/min/mg protein) fall centrally into the range observed with other insects (29), and particularly in other coleopterans such as boll weevil larvae and adults (30) (76–151 pmol/min/mg), western corn rootworm adults (31) (129–285 pmol/min/mg), and yellow mealworm larvae (32) (76 pmol/min/mg). Activity against *p*-nitroanisole (assuming an incubation time of 10 min) likewise is in the mid-range of that found with microsomes from a variety of other insects (33). This shows that the PSMO system in the CPB is well developed. Comparing microsomes from the gut to those from the fat body of last instar larvae, specific activity against aminopyrine in RLI CPB was about threefold higher in the gut, which is again consonant with many other studies on insect microsomes (34, 35). The whole abdomen/thorax was used as the source of microsomes in most of our work, and this result suggests that considerably higher specific PSMO activities would be observed on using isolated gut as a tissue source.

Considerable NADPH oxidase activity was observed, with rates linear for at least 20 min and comparable to those reported for the CPB by Rose and Brindley (28) using combined fat body and gut from last instar larvae. Rates of NADPH oxidation were two- to threefold higher in the two R strains than in the S strain. Although this activity might consist of contributions from several sources, the fact that it is strongly inhibited by PBO pretreatment *in vivo* and by SKF 525-A *in vitro* indicates that the bulk of the NADPH oxidation is being carried out via cytochrome P450. Microsomal NADPH–cytochrome c reductase activity was about 10-fold higher in their study than in ours. This, and the lack of other PSMO reactions, could be due to the apparent partial proteolytic degradation of the reductase in their (28) preparation which renders it incapable of hydrophobic linkage to cytochrome P450, but still capable of reaction with cytochrome c (34).

The subcellular distribution of PSMO activity was studied using aminopyrine N-demethylation as the indicator reaction. Typical of PSMO activity in many other insects (34, 35), activity was located primarily in the microsomal fraction in homogenates of the adult CPB abdomen. Mitochondria and the microsomal supernatant were essentially devoid of activity. Activity was greater in the presence of NADPH than in the presence of NADH. In some cases with insect microsomes, a synergistic response has been reported when these nucleotides were added together (34), but this was not the case in the present study. Although the highest specific activity was found in the microsomal fraction, total activity in this fraction was only 38% of that measured in the 700*g* supernatant. Since to-

tal activity diminishes steadily during the microsomal isolation, it is probable that the PSMO complex is unstable. This was confirmed by the rather high P420:P450 ratios observed in microsomes resuspended in the absence of glycerol. It should be emphasized that the medium and ionic strength of buffers used here are basically those used for house fly microsomes (10). Additional studies pertaining to trial-and-error testing of all the various ingredients and conditions are warranted to develop and preserve PSMOs in CPB.

The relationship between the PSMO activities *in vitro* against the substrates used here and resistance to APM and other insecticides in these strains is unclear. In each R strain, considerable synergism occurs with PBO, indicating an important contribution of PSMOs to resistance. There is a major inequality between relative levels of PSMO activity in the R and S strains (approximately 2- to 3-fold higher), the relative level of PBO synergism (4- to 5-fold higher in the R strain than in the S strain), and the APM resistance levels (180- to 380-fold). A one-to-one relationship between PSMO activity and level of resistance would not necessarily be expected. Overall resistance ratios depend on the total pharmacokinetic relationships in the R and S strains, which are likely to be complex (36). The case of APM toxicity is a particularly difficult one to analyze in regard to PSMO activity, synergism, and resistance since it is probable that competing PSMO reactions either activate the parent compound to the potent anticholinesterase, azinphosmethyloxon, or detoxify APM (25). Further, reactive sulfur released during the conversion to azinphosmethyloxon tends to inhibit the PSMOs, thus limiting the activation reaction and probably inhibiting some isozymes of P450 more than others (37).

Finally, and most important, further study (38) has shown that both the RLI and RM strains have an altered target site (AChE) which also plays a significant role in determining the level of resistance to AChE inhibitors. Resistance is therefore multifactorial. However, our work has established that CPBs contain an array of defensive enzymes against xenobiotics, that these may be elevated in resistant forms, and that the PSMO system is a significant element in the resistance of these strains to APM among a range of different insecticides.

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Mechanisms Associated with Diazinon Resistance in Western Flower Thrips

GUANGYU ZHAO, WEI LIU, AND CHARLES O. KNOWLES[1]

Department of Entomology, University of Missouri, Columbia, Missouri 65211

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Mechanisms of resistance were examined using UMC and KCM western flower thrips, *Frankliniella occidentalis* (Pergande), that differed in their susceptibility to diazinon by 14.2 times, with the latter strain being more tolerant. Diazinon penetration, metabolism, and excretion were faster in KCM than in UMC thrips. Metabolism of diazinon in both strains was mainly oxidative, and major metabolites cochromatographed with diazoxon, 2-isopropyl-4-methyl-6-hydroxypyrimidine, and 2-(2′-hydroxy-2′-propyl)-4-methyl-6-hydroxypyrimidine. Carboxylesterase activity in KCM thrips was significantly lower than that in UMC thrips and was 9.6 and 20.4 times less sensitive to diazoxon and eserine, respectively. Acetylcholinesterase (AChE) activity in KCM and UMC thrips was similar, but that in KCM thrips was 9.6 times less sensitive to diazoxon. Butyrylcholinesterase (BuChE) activity in the two strains was similar and was appreciably higher than AChE activity. BuChE activity in KCM thrips was 170 times less sensitive to diazoxon than that in UMC thrips. No interstrain difference in glutathione *S*-transferase activity was observed with 1-chloro-2,4-dinitrobenzene as substrate. Diazinon resistance in western flower thrips was due chiefly to rapid metabolism and insensitive AChE. The role of BuChE, if any, in diazinon resistance is not currently known; however, by serving as an alternate phosphorylation site, it could reduce the level of organophosphate at the AChE target in both strains. © 1994 Academic Press, Inc.

INTRODUCTION

Western flower thrips (WFT)[2], *Frankliniella occidentalis* (Pergande), cause damage to infested crops by feeding and by transmitting tomato spotted wilt virus (1). The problem is especially severe on ornamentals grown in commercial greenhouses because WFT have developed resistance to organophosphate, carbamate, pyrethroid, and other insecticides (2–4). No studies of the mechanisms of insecticide resistance in WFT were found; therefore, we investigated the pharmacokinetics and pharmacodynamics of diazinon resistance in two strains of WFT.

MATERIALS AND METHODS

Insects. One WFT strain (KCM) was collected in 1989 from Gerbera daisies (varieties Tempo and Festival) at a commercial greenhouse in western Missouri. It was reared on chrysanthemums (variety Charm) in a greenhouse maintained at 24°C during the day and 18°C during the night with a photoperiod of 12:12 (L:D) hr. Bioassays conducted at the time of collection indicated that KCM thrips were resistant to some organophosphates, carbamates, and pyrethroids (2). Beginning in October 1991, KCM thrips were sprayed with diazinon (Diazinon Plus, 25%E, Ortho) about every 45 days. The diazinon concentration was 20 ml per 4 gal of water, and the spray was applied over the top of the plants at a con-

[1] To whom correspondence should be addressed.

[2] Abbreviations used: WFT, western flower thrips; KCM, resistant strain of WFT; UMC, susceptible strain of WFT; AChE, acetylcholinesterase (EC 3.1.3.7); BuChE, butyrylcholinesterase (EC 3.1.1.8); ASCh, acetylthiocholine iodide; BuSCh, butyrylthiocholine iodide; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; CDNB, 1-chloro-2,4-dinitrobenzene; BW 284C51, 1,5-bis(4-allyldimethylammoniumphenyl)-pentane-3-one dibromide; eserine, eserine hemisulfate; DFP, diisopropyl phosphorofluoridate; diazoxon, *O,O*-diethyl *O*-(2-*iso*propyl-6-methyl-4-pyrimidinyl)-phosphate; hydroxypyrimidine, 2-isopropyl-4-methyl-6-hydroxypyrimidine; dihydroxypyrimidine, 2-(2′-hydroxy-2′-propyl)-4-methyl-6-hydroxypyrimidine.

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stant pressure of 90 psi. Mortality of KCM thrips was high initially (>60%) and decreased with subsequent applications of diazinon. The other strain (UMC) was collected in February 1992 from chrysanthemums in a greenhouse at the University of Missouri and reared as described above, except that no insecticide selection pressure was applied, however, soap (Safer, Safer, Inc.) was used occasionally to control aphids. UMC thrips possessed low levels of resistance to some organophosphate, carbamate, and pyrethroid insecticides at the time of collection. Adult vial bioassays conducted in 1993 revealed that the LC_{50}'s (95% CL) for diazinon were 17.5 (12–28) and 249 (182–385) μg/vial for UMC and KCM WFT, respectively, compared with 1.97 (1.2–3.0) μg/vial determined for the reference strain in 1991 (Knowles *et al.*, unpublished).

Chemicals. Samples of analytical grade diazinon (96.7%) and [2-^{14}C]diazinon-pyrimidine (sp act 11.32 mCi/mmol) were provided by Ciba-Geigy Chemical Co. (Greensboro, NC). TLC of [^{14}C]diazinon on silica gel-coated plates (250 μm, LK4DF, Whatman) with *n*-hexane–acetone (4:1) (5) yielded a radiochemical purity of 95% upon radioscanning (Rita-3200 TLC Analyzer; Raytest, McMurray, PA). Diazoxon, hydroxypyrimidine, and dihydroxypyrimidine also were provided by Ciba-Geigy. Average R_f values in the above-mentioned solvent system were 0.48, 0.19, 0.13, and 0.05 for diazinon, diazoxon, hydroxypyrimidine, and dihydroxypyrimidine, respectively. ASCh, BuSCh, DTNB, α-naphthyl acetate, sodium lauryl sulfate, Fast Blue RR, CDNB, glutathione, eserine, BW 284C51, and DFP were obtained from Sigma Chemical Co. (St. Louis, MO); methomyl was from DuPont, Inc. (Wilmington, DE).

Pharmacokinetic studies. About 40 mg of adult WFT (about 700 thrips), selected without regard to sex or age, was placed in each of 12 capped glass scintillation vials (20 ml) that had been precoated with about 100,000 dpm (about 1.12 μg) of a methylene chloride solution of [^{14}C]diazinon. Three replicates were analyzed after exposure times of 1, 6, 12, or 24 hr. WFT were transferred to a funnel fitted with filter paper (Whatman No. 2, 5.5 cm) and rinsed with methanol (3 × 10 ml). An aliquot of the combined external rinses was radioassayed in 10 ml of ScintiVerse BD (Fisher Scientific Co., St. Louis, MO) with a Beckman LS 7500 liquid scintillation counter; the remainder was reduced in volume under a stream of dry nitrogen and subjected to TLC and radioscanning as described above. The filter paper was allowed to dry, cut into pieces, and radioassayed in ScintiVerse. The total radiocarbon in the insect rinse consisted of the sum of that in the methanol rinse and filter paper. WFT were extracted by homogenizing in acetone (3 × 5 ml). An aliquot was radioassayed to determine total internal radiocarbon, and the remainder was concentrated and subjected to TLC. To the holding container was added acetone (5 ml); an aliquot was radioassayed to estimate total radiocarbon in container rinse, and the remainder was subjected to TLC.

Enzyme assays. For assay of carboxylesterases, WFT were homogenized in 0.4 *M* phosphate buffer (pH 7.0), and the homogenate was centrifuged at 450*g* for 10 min. Carboxylesterase activity in the 450*g* supernatant was measured by the method of van Asperen (6). Briefly, 1.0 ml of enzyme preparation equivalent to 0.5 thrips and 10 μl of α-naphthyl acetate (0.5 m*M* final) were incubated in a water bath at 30°C for 15 min. One milliliter of diazoblue lauryl sulfate solution was added, and the absorbance at 600 nm (Milton Roy Spectronic 1201) was read after 15 min. Hydrolysis of α-naphthyl acetate was linear up to the equivalent of 0.75 WFT per tube. AChE and BuChE activities in the 110,000*g* pellet and supernatant, respectively, of WFT homogenates were assayed by the Gorun *et*

al. (7) modification of the method of Ellman *et al.* (8) as described by Liu *et al.* (9). The reaction mixture consisting of 160 μl of enzyme preparation equivalent to six thrips (ASCh) or 1.5 thrips (BuSCh) and 40 μl of substrate (1.0 m*M* final) was incubated in a water bath at 37.5°C for 15 min (ASCh) or 5 min (BuSCh) prior to addition of 1.8 ml of DTNB reagent. The absorbance at 412 nm was read immediately.

To study inhibition of carboxylesterase and cholinesterase activities, aliquots of potential inhibitors dissolved in distilled water or acetone at appropriate concentrations were added to the tubes. (When acetone was used, it was allowed to evaporate before the enzyme preparation was added.) Inhibitor and enzyme preparation were incubated for 15 min at 25°C prior to addition of the substrate.

Glutathione *S*-transferase activity was assayed using the model substrate CDNB as described previously (10, 11). The reaction mixture consisting of 200 μl of 10,000*g* supernatant equivalent to six WFT, 2.0 ml of glutathione (5.0 m*M* final), 80 μl of CDNB in acetone (1.0 m*M* final), and 720 μl of 0.1 *M* phosphate buffer (pH 6.5) was placed in a cuvette, and the absorbance at 340 nm was recorded after 5 min. Activity was directly proportional to enzyme concentration.

Corrections for nonenzymatic degradation of substrate and endogenous enzyme interference were made when appropriate in enzyme assays.

Electrophoresis studies. Aliquots of 450*g* supernatants of WFT homogenates prepared in 0.1 *M* phosphate buffer were subjected to electrophoresis in native gradient (8–25%) polyacrylamide as described by Liu *et al.* (9). Gels were cut into strips and placed in 0.1 *M* phosphate buffer (pH 6.5) (controls) or in buffer solutions of BW 284C51 (100 μ*M*), eserine (100 μ*M*), DFP (1.0 μ*M*), or diazoxon (1.0 μ*M*). Hydrolysis of α-naphthyl acetate was detected as described by Hunter and Burstone (12); in some cases gels were soaked in 0.5 *M* boric acid (pH 4.1) for 30 min to lower the pH of the gel and accelerate the chromogenic reaction (13).

RESULTS

Pharmacokinetic studies. The distribution and nature of radiocarbon following exposure of WFT to [^{14}C]diazinon are presented in Fig. 1 and Table 1. After 1 hr of exposure to diazinon, total levels of radiocarbon in the container rinse and insect rinse were 53.2 and 29.5%, respectively, for UMC thrips and 48.0 and 33.4%, respectively, for KCM thrips (Fig. 1). Thus, total external radiocarbon levels after 1 hr were 82.7 and 81.4% for UMC and KCM thrips, respectively. Of this external radiocarbon, 70.3% was diazinon with UMC thrips and 61.2% was diazinon with KCM thrips (Table 1). Therefore, during the initial hour of exposure diazinon penetrated into KCM thrips somewhat faster than into UMC thrips. Also, the presence of nonparent radiocarbon in the container and insect rinses at 1 hr (Table 1) indicated that metabolism and excretion were occurring because diazinon was stable in the vials for at least 24 hr in the absence of WFT (data not shown). Levels of diazinon and metabolites 2 and 4 were higher in the internal fraction of UMC thrips than in KCM thrips, whereas levels of metabolite 3 were higher in KCM thrips.

The greatest interstrain difference in diazinon pharmacokinetics was observed between 1 and 6 hr. In the case of UMC thrips, there was little change in the distribution and nature of radiocarbon in the container rinse, insect rinse, and internal fraction from that found at 1 hr (Figs. 1 and 2, Table 1). However, in the case of KCM thrips, there was a marked increase in total levels of radiocarbon in the container rinse and a decrease in levels of radiocarbon in the insect rinse. A slight decrease in total levels of radiocarbon in the internal fraction of KCM thrips during this time interval also was observed. These changes in distribution of radiocarbon in KCM thrips were ac-

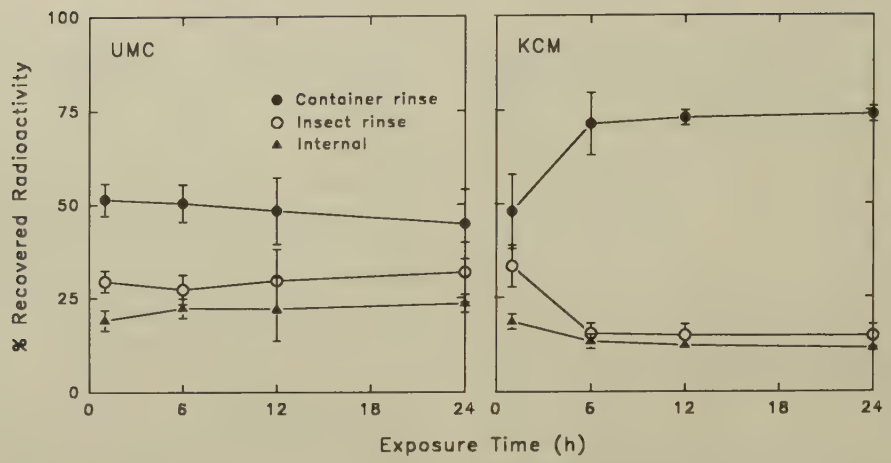

FIG. 1. *Distribution of radiocarbon during exposure of UMC and KCM western flower thrips to [^{14}C]diazinon. Data are means (n = 3); SD value given when greater than size of symbol. Recovery of radiocarbon was 94.7 ± 0.04% (n = 15) and 95.2 ± 0.03% (n = 15) for UMC and KCM experiments, respectively.*

companied by changes in the nature of the radiocarbon (Fig. 2, Table 1). In the container rinse, there was a marked decrease in the levels of diazinon that was accompanied by marked increase in levels of metabolites 3 and 4 (Table 1). The diazinon level in the insect rinse also decreased and was associated with increased levels of metabolite 3 (Table 1).

Between 6 and 12 hr, the major change in diazinon pharmacokinetics was associated with UMC thrips and was manifested by a decrease in diazinon levels in the container rinse, increases in metabolites 3 and 4 in the container rinse, and increase in metabolite 3 in the insect rinse. The distribution and nature of radiocarbon in the container rinse, insect rinse, and internal fraction of UMC and KCM thrips at 24 hr were not substantially different from those observed at 12 hr.

Total levels of diazinon decreased from 75.2% at 1 hr to 35.5% at 24 hr with UMC thrips and from 62.7% at 1 hr to only 1.3% at 24 hr with KCM thrips (Table 1). Diazinon metabolites tentatively identified by cochromatography included diazoxon (metabolite 2), hydroxypyrimidine (metabolite 3), and dihydroxypyrimidine (metabolite 4).

Carboxylesterase assays. Preparations from UMC thrips hydrolyzed α-naphthyl acetate faster than did those from KCM thrips; rates of hydrolysis (n = 12) were 1000 ± 24 and 716 ± 15 nmol/mg protein/min for UMC and KCM thrips, respectively. α-Naphthyl acetate hydrolysis by UMC thrips was more sensitive to inhibition by diazoxon, eserine, and methomyl than was that by KCM thrips (Fig. 3, Table 2). BW 284C51 at 100 μ*M* gave no detectable inhibition of α-naphthyl acetate hydrolysis by KCM thrips (Table 2).

Electrophoresis of preparations from UMC thrips revealed the presence of carboxylesterase activity at the origin and eight bands that could be separated into two groups, with group A consisting of the three slowest migrating bands and group B consisting of the five fastest migrating bands (Fig. 4). Preparations from KCM thrips contained α-naphthyl acetate activity at the origin and the group B esterases; however, group A esterases were not detected. Except for weak activity remaining at the origin, DFP abolished hydrolysis of α-naphthyl acetate by preparations from both strains. Diazoxon abolished α-naphthyl acetate hydrolysis by preparations

TABLE 1

Distribution and Nature of Radiocarbon after Exposure of UMC and KCM Strains of Western Flower Thrips to [^{14}C]Diazinon

Distribution and nature of radiocarbon	% Radiocarbon after indicated hour of exposure[a]			
	1	6	12	24
		UMC		
Container rinse				
Diazinon	46.1 (1.3)	41.4 (2.8)	24.3 (2.8)	21.0 (2.3)
Metabolite 3[b]	2.7 (0.7)	5.8 (2.3)	16.0 (1.9)	16.0 (1.6)
Metabolite 4	0.8 (0.3)	1.8 (0.1)	6.6 (1.4)	7.1 (1.2)
Remainder	3.6 (0.7)	2.9 (0.4)	2.8 (0.4)	1.5 (0.3)
Insect rinse				
Diazinon	24.2 (2.1)	18.1 (1.3)	11.3 (1.5)	10.8 (1.3)
Metabolite 2	2.6 (0.8)	2.2 (0.2)	2.6 (0.1)	3.3 (0.1)
Metabolite 3	1.4 (0.3)	4.2 (1.1)	11.9 (1.4)	14.1 (0.2)
Metabolite 4	1.1 (0.2)	2.2 (0.7)	3.8 (1.1)	2.8 (0.5)
Remainder	0.2 (0.1)	0.6 (0.3)	<0.1 (0.2)	0.8 (0.2)
Internal				
Diazinon	4.9 (0.9)	4.4 (1.6)	3.2 (0.9)	3.7 (0.7)
Metabolite 2	5.5 (1.6)	6.0 (0.9)	5.1 (0.1)	7.1 (1.2)
Metabolite 3	7.2 (1.8)	9.9 (2.2)	10.8 (0.4)	10.0 (0.3)
Metabolite 4	1.5 (0.6)	1.8 (0.4)	2.4 (0.2)	2.7 (1.2)
Remainder	<0.1 (0.2)	0.2 (0.1)	0.6 (0.2)	<0.1 (0.1)
		KCM		
Container rinse				
Diazinon	33.7 (1.7)	8.6 (1.6)	1.1 (0.5)	0.4 (0.1)
Metabolite 3[b]	9.6 (1.2)	49.1 (3.1)	58.0 (0.7)	57.2 (0.4)
Metabolite 4	1.8 (0.2)	9.6 (0.9)	12.2 (0.4)	14.4 (0.5)
Remainder	2.9 (0.9)	4.0 (1.1)	1.6 (0.7)	1.8 (0.9)
Insect rinse				
Diazinon	27.5 (0.8)	2.6 (0.2)	2.1 (0.1)	0.7 (0.1)
Metabolite 3[b]	2.5 (0.5)	11.0 (0.1)	10.8 (0.3)	11.9 (0.1)
Metabolite 4	1.7 (0.2)	1.2 (0.1)	1.8 (0.1)	1.7 (0.2)
Remainder	1.7 (0.2)	0.6 (0.1)	0.2 (0.3)	0.4 (0.3)
Internal				
Diazinon	1.5 (0.1)	0.7 (0.1)	0.6 (0.2)	0.2 (0.0)
Metabolite 3[b]	15.1 (0.4)	11.7 (0.1)	10.0 (0.3)	9.7 (0.4)
Metabolite 4	0.6 (0.1)	0.5 (0.1)	0.7 (0.1)	1.6 (0.2)
Remainder	0.5 (0.2)	0.4 (0.2)	0.9 (0.2)	<0.1 (0.1)

[a] Values are means (±SD); $n = 3$ except for UMC 12-hr insect rinse for metabolite 3 where $n = 2$.

[b] May contain small amount of metabolite 2.

from both strains, except for the two intense bands of activity in group B which were apparently unaffected. Eserine gave some inhibition of activity at the origin and appeared to reduce the intensity of some of the bands in group B. BW 284C51 inhibited activity only at the origin with preparations from both strains.

Cholinesterase assays. When chrysanthemums infested with KCM thrips were sprayed with diazinon, AChE activity was rapidly depressed, reaching about 25% of pretreatment levels at 4 days (Fig. 5). BuChE activity was completely depressed by 2 days after treatment. Both AChE and BuChE activity slowly recovered, reaching pretreatment levels between 20 and 24 days after spraying.

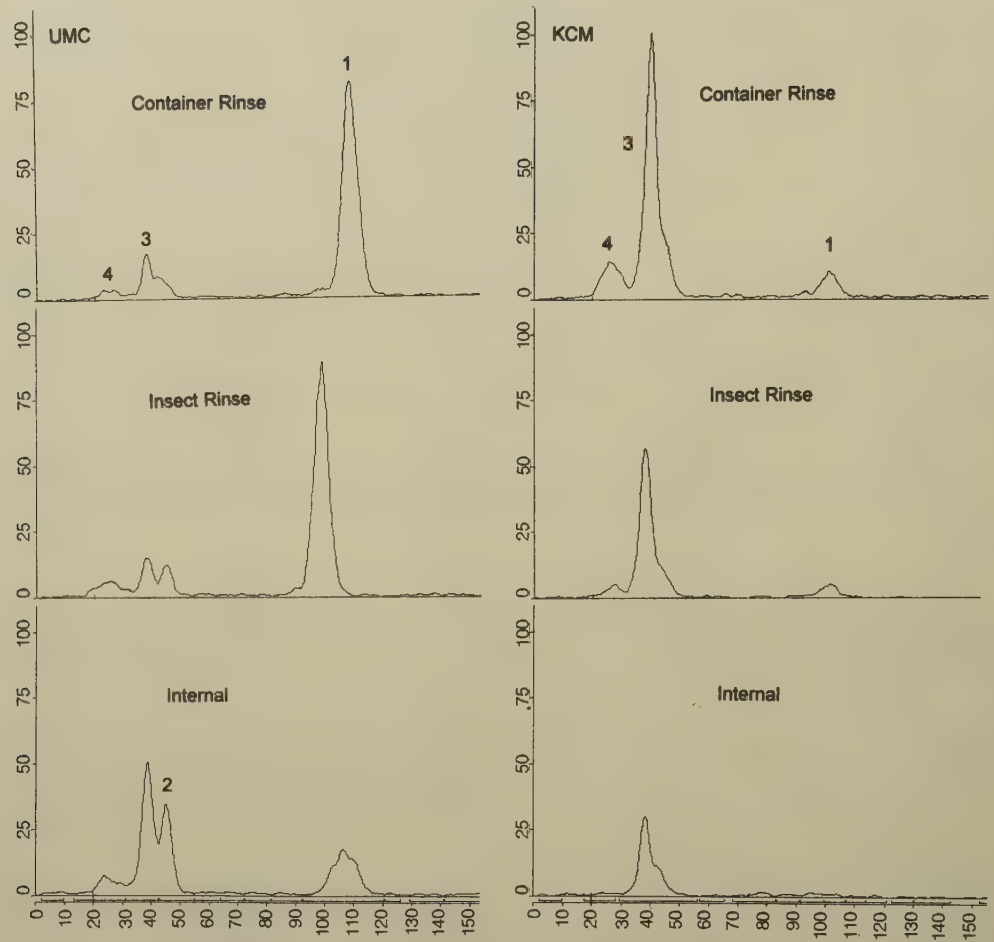

FIG. 2. *Representative radiochromatogram scans of fractions from UMC and KCM western flower thrips after 6 hr of continuous exposure to [^{14}C]diazinon. Ordinate ranges from 0 to 100 counts; abscissa ranges from 0 to 150 mm. Compound 1 is diazinon; compounds 2–4 are metabolites.*

AChE activities (n = 3) were 129 ± 5 and 121 ± 10 nmol/mg protein/min for UMC and KCM thrips, respectively, whereas BuChE activities were 765 ± 107 and 832 ± 87 nmol/mg protein/min for UMC and KCM thrips, respectively. Diazoxon pI_{50}'s were 6.48 and 5.50 for inhibition of ASCh hydrolysis by UMC and KCM preparations, respectively, and pI_{50}'s were 12.09 and 9.86 for inhibition of BuSCh hydrolysis by UMC and KCM thrips, respectively (Fig. 6).

Glutathione S-transferase assays. With CDNB as substrate, there was no significant interstrain difference in glutathione *S*-transferase activity. The activity of UMC preparations (n = 3) was 261 ± 11 nmol/mg protein/min, whereas that for KCM preparations was 260 ± 11 nmol/mg protein/min.

DISCUSSION

This study used two strains of WFT. UMC thrips possessed a low level (8.9 times) of resistance to diazinon, whereas KCM thrips, which were reared with diazinon selection pressure, possessed a relatively high level (126.4 times) of resistance to this organophosphate. The toxicity of diazinon to KCM thrips was synergized by piperonyl butoxide but not by *S,S,S*-tributylphosphorotrithioate (Knowles *et*

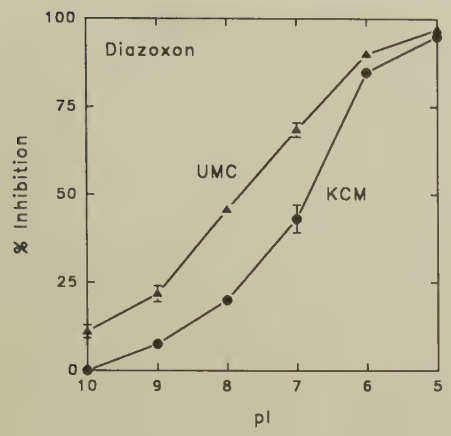

FIG. 3. *Effect of diazoxon concentration of the hydrolysis of α-naphthyl acetate (0.5 mM) by the 450g supernatant of UMC and KCM western flower thrips. pI, negative logarithm of inhibitor concentration in mol/liter. Data are means (n = 3); SD value given when greater than size of symbol.*

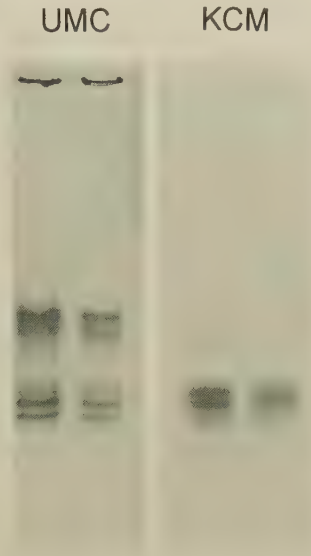

FIG. 4. *Nondenaturing polyacrylamide gel electrophoresis of two different concentrations (about 5.0 and 1.25 μg total protein) of 450g supernatants of UMC and KCM western flower thrips with α-naphthyl acetate as substrate.*

al., unpublished results), suggesting that enhanced metabolism of diazinon by oxidative rather than hydrolytic reactions was important in conferring resistance.

Pharmacokinetic studies indicated that diazinon penetration, metabolism, and excretion were faster in KCM thrips than in UMC thrips. The rapid penetration of diazinon in KCM thrips likely resulted from the fast metabolism and excretion of [^{14}C]diazinon equivalents. Diazinon metabolites tentatively identified by cochromatography included diazoxon, hydroxypyrimidine, and dihydroxypyrimidine; other polar unidentified metabolites were also present. These three metabolites of diazinon and their mode of formation in insects have been reported previously. For example, diazoxon was formed by oxidative desulfuration of diazinon by preparations of house flies, *Musca domestica* L., and American cockroaches, *Periplaneta americana* (L.) (14–

TABLE 2

Effect of Inhibitors on the Hydrolysis of α-Naphthyl Acetate by Esterases in Homogenates of Western Flower Thrips

Inhibitor	pI_{50} (mol/liter) UMC	KCM	Selectivity
Diazoxon	7.80	6.82	9.55
DFP	7.61	7.62	(1.02)
Eserine	5.59	4.28	20.42
Methomyl	4.82	4.61	1.62
BW 284C51	—	0[a]	—

[a] No inhibition was observed at a concentration of 100 μ*M*.

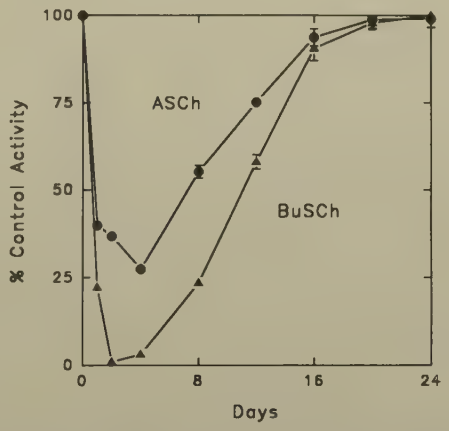

FIG. 5. *Cholinesterase activity in 450 supernatants of KCM western flower thrips at various intervals following spraying infested chrysanthemums with diazinon. Data are means (n = 3); SD value given when greater than size of symbol.*

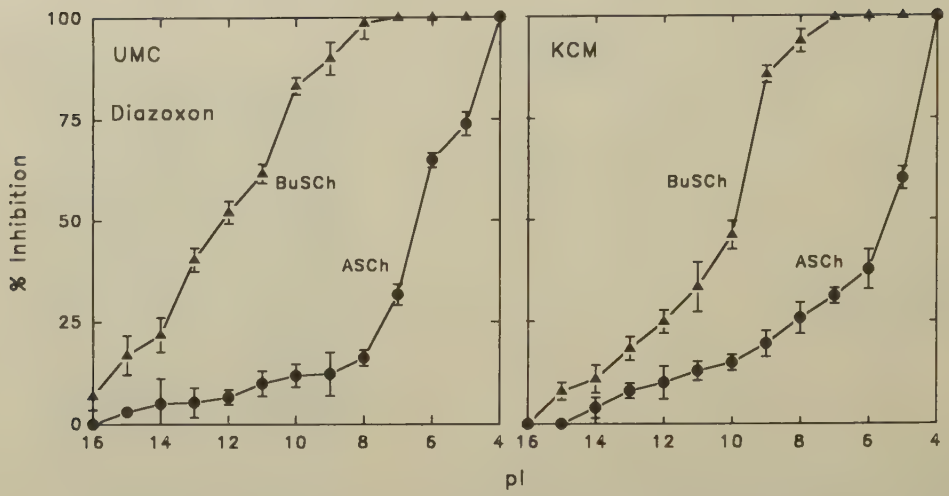

FIG. 6. *Effect of diazoxon concentration on hydrolysis of acetylthiocholine and butyrylthiocholine (1.0 mM) by the 110,000g pellets (ASCh) and supernatants (BuSCh), respectively, of UMC and KCM western flower thrips. Data are means (n = 3); SD value given when greater than size of symbol.*

19). In house fly and American cockroach preparations, hydroxypyrimidine was formed by oxidative cleavage of diazinon and diazoxon at the aryl ester linkage and by hydrolytic cleavage of diazoxon (15, 16, 19). Dihydroxypyrimidine was formed in American cockroach preparations by oxidative cleavage of hydroxydiazinon and hydroxydiazoxon that were produced from diazinon and diazoxon, respectively, by oxidation at carbon No. 2 of the isopropyl moiety of the pyrimidine ring (16). Thus, in WFT diazoxon likely was formed by oxidative desulfuration of diazinon, and hydroxypyrimidine probably was formed by oxidative cleavage of diazinon or diazoxon or both. The mode of formation of dihydroxypyrimidine is difficult to discern because the hydroxydiazinon and hydroxydiazoxon intermediates were not identified. Hydroxydiazoxon may have been one of the unidentified metabolites; however, no unknown [^{14}C]diazinon metabolite from WFT was observed chromatographing in the region reported for hydroxydiazinon (16). Thus, dihydroxypyrimidine could have formed from hydroxydiazoxon or more likely from oxidation of hydroxypyrimidine.

Several metabolic resistance mechanisms have been reported for diazinon in insects, including cleavage of diazinon to diethyl phosphorothioic acid and hydroxypyrimidine by oxidases, cleavage of diazoxon to diethyl phosphoric acid and hydroxypyrimidine by phosphotriesterases, and dealkylation and dearylation of diazinon and diazoxon by glutathione *S*-transferases (15, 18–20). Although oxidative desulfuration of diazinon to diazoxon is an activation reaction, it was also found to be enhanced in some resistant house flies (14); however, its toxic action was negated by some of the detoxification mechanisms mentioned above. Of the oxidases, phosphotriesterases, and glutathione *S*-transferases, oxidases were most involved with diazinon metabolism in WFT and were most important in conferring metabolic resistance, although WFT possessed esterase and glutathione *S*-transferase activities.

Homogenates of UMC and KCM thrips hydrolyzed α-naphthyl acetate, a substrate that is cleaved in insect preparations mainly by carboxylesterases (aliesterases) and AChEs (6). Further, activity toward α-naphthyl acetate was appreciably higher in homogenates of UMC thrips than those of KCM thrips. However, no interstrain

difference in activity toward ASCh was detected, an observation in agreement with that of Liu *et al.* (9). Therefore, the interstrain difference in activity toward α-naphthyl acetate was associated with carboxylesterase and not AChE activity. Electrophoretic studies revealed that fewer carboxylesterases were present in KCM thrips than in UMC thrips. Also, they indicated that activity toward α-naphthyl acetate at the electrophoretic origin was sensitive to eserine and BW 284C51 and thus was due to AChE. Liu *et al.* (9) reported that AChE activity in homogenates of UMC and KCM thrips was sensitive to eserine and BW 284C51 and would not enter the gel in the absence of detergent.

It is of interest that the more diazinon-resistant KCM thrips had lower total carboxylesterase activity and fewer carboxylesterases than the more susceptible UMC thrips, although the difference was only 1.4-fold. Much lower levels of carboxylesterase activity, as assayed with several substrates, including methyl butyrate and α-naphthyl acetate, were found in homogenates of several organophosphate-resistant strains of house flies when compared with those of susceptible strains (6, 21, 22) and in some other insects (29). It was thought to be associated with organophosphate resistance including resistance to diazinon (6, 22). However, high levels of carboxylesterase activity have been associated with organophosphate resistance in some insects, where they have usually been linked with enhanced degradation of the insecticide (23–28). There was no evidence that carboxylesterases in UMC and KCM thrips hydrolyzed diazoxon because activity in both strains was inhibited greater than 95% by 10 μ*M* concentrations of the phosphate. The fact that α-naphthyl acetate hydrolysis by KCM thrips was 9.6 times less sensitive to diazoxon than that by UMC thrips was likely due to the presence in KCM homogenates of higher levels of insensitive AChE as discussed below. Thus, it was concluded that carboxylesterases were not involved in the resistance of these WFT to diazinon. This conclusion was supported by results from the pharmacokinetic studies that indicated diazinon metabolism in UMC and KCM thrips was chiefly oxidative and not hydrolytic in nature.

As mentioned above, homogenates of UMC and KCM thrips did not differ in their hydrolytic activity toward ASCh; however, ASCh hydrolysis in KCM thrips was 9.6 times less sensitive to diazoxon than that in UMC thrips. Liu *et al.* (9) found that homogenates of UMC and KCM thrips each contained two forms of AChE based on biphasic BW 284C51 concentration-inhibition curves. Also, they concluded that the more sensitive form predominated in homogenates of UMC thrips, whereas the more resistant form predominated in homogenates of KCM thrips (9). It seemed reasonable to suggest that the interstrain difference in sensitivity of ASCh hydrolysis to diazoxon was due to the presence in KCM thrips of higher levels of the insensitive AChE and that the diazoxon-insensitive AChE contributed to the diazinon resistance. Insensitive AChE has been implicated as an organophosphate and carbamate resistance mechanism in numerous insects and acarine species (29, 30).

An interesting observation was that the hydrolysis of BuSCh in homogenates of KCM thrips was 170 times less sensitive to diazoxon than that of UMC thrips. Liu *et al.* (9) observed that hydrolysis of BuSCh by homogenates of UMC and KCM thrips was eserine and DFP sensitive and BW 284C51 insensitive. Thus, it was concluded that BuSCh hydrolysis by UMC and KCM thrips was catalyzed by BuChE (9). Clearly, BuChE activity was affected more by diazinon selection pressure than was AChE activity. Because the function of BuChE in insects and higher animals is unknown (31), it is not currently possible to explain the significance of this observatio However, the BuChE is an alternate ph

phorylation site for diazoxon, and it may be serving as a scavenger enzyme to reduce the levels of organophosphate at the AChE target in both strains as suggested by Liu *et al.* (9).

There was no significant interstrain difference in the levels of glutathione *S*-transferase activity as assayed with CDNB. Although it is recognized that CDNB may not always be the best substrate for assay of those enzymes acting on insecticides (32), the pharmacokinetic studies did not suggest that glutathione *S*-transferase played a major role in diazinon metabolism by WFT.

It was concluded that diazinon resistance in WFT was due to a combination of rapid metabolism, which was chiefly oxidative, and insensitive AChE. It has been shown that reduced AChE sensitivity strongly interacts with detoxification, causing a much higher resistance than when present separately (11, 33).

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Mixed-Function Oxidase and Glutathione *S*-Transferase Activities from Field-Collected Larval and Adult Tobacco Budworms, *Heliothis virescens* (F.)

M. L. KIRBY,[1] R. J. YOUNG, AND J. A. OTTEA

Department of Entomology, Louisiana State University Agricultural Center, Louisiana Agricultural Experiment Station, Baton Rouge, Louisiana 70803

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Mixed-function oxidase and glutathione *S*-transferase activities were measured from tissue homogenates of larval and adult tobacco budworms that were collected from cotton fields in Texas and Louisiana. Mixed-function oxidase activity toward *p*-nitroanisole was detected in both developmental stages and, in six of the eight field collections tested, mean levels of activity were significantly higher than activity measured in parallel assays with insects from a laboratory-reared, insecticide-susceptible colony. However, the significance of this activity with respect to pyrethroid metabolism and resistance is unclear because variation in mixed-function oxidase activity among these strains was poorly correlated with susceptibility to cypermethrin. Glutathione *S*-transferase activities toward 3,4-dichloronitrobenzene and 1-chloro-2,4-dinitrobenzene were also detected in both larvae and adult insects, and mean levels varied among the field collections tested. Patterns of activity in tissues examined for both mixed-function oxidases and glutathione *S*-transferases were different between larvae and adults suggesting that the regulation and expression of these enzyme activities is dependent on developmental stage. © 1994 Academic Press, Inc.

INTRODUCTION

Mixed-function oxidases (EC 1.14.14.1; MFOs)[2] and glutathione *S*-transferases (EC 2.5.1.18; GSTs) enhance survival of phytophagous insects by detoxifying a wide array of xenobiotics including insecticides and plant allelochemicals [reviewed in (1, 2)]. High levels of MFO and GST activities are measured in insects that are resistant to insecticides (3–6) and that feed on plants containing toxins (7–13).

Whereas a number of biochemical studies have examined MFO and GST activities in immature stages of lepidopteran pests (8, 14–18), there are relatively few reports where activities were measured from adults (19, 20). Because of dramatic differences in the life histories between developmental stages of holometabolous insects, comparing patterns of expression for MFO and GST activities in larvae and adults may provide insight into the involvement of these enzymes in the metabolism of host plant allelochemicals (21) and endogenous substrates (22, 23). In addition, many questions concerning the potential for insecticide metabolism by MFOs and GSTs in adult pest insects remain unanswered.

In this report, MFO and GST activities toward noninsecticidal substrates were measured in field-collected larval and adult tobacco budworms, *Heliothis virescens* (F.). This insect is a major pest of cotton grown in the southeastern United States (24) and has the potential to develop high levels of metabolic resistance to insecticides (3, 25–27). Patterns of expression for both enzymes in field-collected strains were different between larval and adult tobacco budworms, suggesting differences in the regulation of these activities between developmental stages of this insect. In ad-

[1] Present address: Department of Entomology, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061.

[2] Abbreviations used: MFO, mixed-function oxidase; GST, glutathione *S*-transferase; PNA, *p*-nitroanisole; DCNB, 3,4-dichloronitrobenzene; CDNB, 1-chloro-2,4-dinitrobenzene.

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dition, although MFO and GST activities toward the substrates examined were higher in resistant than susceptible strains, levels of enzyme activities in insecticide-resistant collections were not correlated with susceptibilities to a pyrethroid insecticide. This result is inconsistent with a role of these enzyme activities measured in this study in insecticide metabolism and illustrates the limitations of using activities toward noninsecticidal substrates as indicators of metabolic resistance to insecticides.

MATERIALS AND METHODS

Chemicals

Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, bovine serum albumin (fraction 5), nicotinamide adenine dinucleotide phosphate, *p*-nitroanisole (PNA), and *p*-nitrophenol were purchased from Sigma Chemical Co. (St. Louis, MO). Phenylthiourea, trichloroacetic acid, potassium chloride, 3,4-dichloronitrobenzene (DCNB), and 1-chloro-2,4-dinitrobenzene (CDNB) were from Aldrich Chemical Co. (Milwaukee, WI).

Insects

Leaves containing *H. virescens* eggs were collected at random from cotton plants grown on Louisiana State University's Northeast Research Station/Macon Ridge Station (MRS; Winnsboro, LA) during June, and the Red River Research Station (RR; Bossier City, LA) during June (JU), July (JLY), and August (AUG) 1992. In addition, four cotton growing regions of Texas that have a history of heavy insecticide use and that experienced control difficulties during 1992 were sampled during July (Harlingen; HARL) and August (Uvalde; UVLD, Hearne; HNE and Snook; SNK). Susceptibilities of these strains to a number of insecticides have been measured and results are reported elsewhere (28). Eggs (200–500) were transferred from collection sites to the laboratory in styrofoam coolers containing ice, and leaves containing eggs were placed in 1-oz cups containing a pinto bean-based artificial diet at 27°C, LD 14:10, and 70% relative humidity (29). An insecticide-susceptible strain (LSU) of *H. virescens* has been in continuous culture for 15 years without insecticide exposure (29) and was used as a reference strain for biochemical and toxicological tests. Larvae from field collections were identified as *H. virescens* using a mandible morphology as a criterion (30), and segregated after head capsule slippage during the final day of the fourth instar. The following day, insects that had completed molting and commenced feeding were used for experiments. Gender of larvae was determined during dissection (see below) by the presence or the absence of pale yellow testicular sacks. Adult insects were reared in one gallon ice cream containers, provided with a 10% sucrose:water solution as a carbohydrate source, and segregated by sex 1 day following emergence. Mean weights of test insects ranged from 0.124 to 0.164 g (larvae) and 0.129 to 0.201 g (adults).

Enzyme Assays

Developmentally synchronous larvae (fifth-instar, Day 1) or moths (1 day post-emergence) were weighed, and tissues from individual insects were homogenized in 500 μl (for larval midguts), 300 μl (for larval fat bodies), or 1 ml (for adult abdomens) of ice-cold 1.15% KCl (containing a few crystals of phenylthiourea) using 10 strokes of an all-glass homogenizer. This, and subsequent manipulations of homogenates (unless otherwise indicated), were made at 0–4°C. Homogenates were transferred to centrifuge tubes and spun at 4°C for 10 min at 10,000*g*. Resulting supernatants were used for assays within 30 min of preparation.

Larval and adult MFO activities from individual midguts (larvae) or whole abdomens (adults) were quantitated using the assay of Kinoshita *et al.* (31) as modified by Hansen and Hodgson (32) and Shang and Soderlund (16). In preliminary experi-

ments, larval MFO activities were also detected in pooled (i.e., multiple insect) homogenates of fat body and carcass tissues, but activities measured from tissue homogenates of individual insects were very near to (or below) the limit of detection of this assay (26.9 pmol/min). Assay conditions used to measure MFO activities were optimized with respect to incubation time and protein content (data not shown). Duplicate reaction mixtures contained (concentrations in final volume of 200 μl): enzyme homogenate, phosphate buffer (0.1 *M*, pH 7.7), and an NADPH-generating system consisting of NADP (1.18 m*M*), glucose-6-phosphate (24 m*M*), and glucose-6-phosphate dehydrogenase (0.5 units). Volumes of enzyme homogenate used corresponded to (mean ± SD) 0.17 ± 0.05 mg protein (0.11 ± 0.01 tissue equivalents) and 0.12 ± 0.04 mg protein (0.04 ± 0.01 tissue equivalents) for larval and adult assays, respectively. Incubations containing only protein and buffer were used as nonenzymatic blanks. Reactions were initiated by adding 2 μl PNA (in ethanol, 0.35 m*M* final concentration), incubated at 27°C for 40 min, then stopped by adding 10 μl trichloroacetic acid (2.5% final) with vigorous mixing. Tubes were centrifuged at 10,000*g* for 10 min, and supernatants (190 μl) were carefully removed and added to individual wells of a flat-bottomed, microtiter plate (Costar, Cambridge, MA) containing 10 μl of 5 *N* sodium hydroxide. Plates were shaken and the absorbance in each well was measured at 405 nm using a Thermomax (Molecular Devices) microplate reader. Absorbance values were corrected for nonenzymatic activity and converted to pmol *p*-nitrophenol formed/min using an experimentally derived "extinction coefficient" of 10.41 (200 μl^{-1} mM^{-1}).

Measurements of GST activities toward DCNB and CDNB were made using the assays of Booth *et al.* (33) and Jakoby (34), respectively, as modified by Grant *et al.* (35, 36). Optimal pH and protein concentrations for GST measurements with these substrates were estimated on the basis of four determinations (data not shown). Buffered substrate solutions were prepared by mixing stock solutions of either DCNB or CDNB (50 m*M* in DMSO) and phosphate buffer (0.1 *M*, pH 8.0 containing 15% glycerol). Reaction mixtures were incubated in flat-bottom microtiter plates (Costar) and included 200 μl of either DCNB or CDNB substrate buffer (0.5 m*M* final concentration), enzyme homogenate, and double-distilled water to make a prereaction volume of 270 μl. Volumes of larval homogenate used corresponded to (mean ± SD) 0.03 ± 0.01 mg protein (0.13 ± 0.01 tissue equivalents) and 0.05 ± 0.01 mg protein (0.16 ± 0.04 tissue equivalents) for assays with CDNB and DCNB, respectively. For adults assays, homogenate volumes corresponded to (mean ± SD) 0.07 ± 0.03 mg protein (0.03 ± 0.01 tissue equivalents) and 0.07 ± 0.03 mg protein (0.04 ± 0.01 tissue equivalents) for assays with CDNB and DCNB, respectively. Reactions were initiated by adding 30 μl GSH (8 m*M* final concentration, in water), and rate of change in optical density at 340 nm during the initial 10 min of the reaction was measured and converted to nmol/min using the experimentally derived extinction coefficients of 8.5 or 10.09 for DCNB or CDNB, respectively (35, 36). Activities were corrected for nonenzymatic metabolism using incubations without protein as controls.

MFO and GST activities are expressed as product formed min^{-1} tissue $equivalent^{-1}$ (mean ± SD) based on duplicate determinations of 19–66 (larval) or 10–20 (adult) homogenates prepared from individual insects. Pairwise comparisons of activities between strains were made using Student's *t* test ($\alpha = 0.01$). Protein concentrations in reaction mixtures were estimated by the method of Bradford (37) using bovine serum albumin (fraction 5; concentrations corrected for impurities) as the standard.

RESULTS

Larval MFO activities varied among the

strains tested (Fig. 1). Relatively low levels of activity were measured for the insecticide-susceptible LSU strain and the early season (June) field collections from the Macon Ridge and Red River Research Stations. Activities for the remaining collections were significantly higher (2.4 to 4.6-fold) than those measured in the LSU strain and ranged from 50.8 (for RR-JLY) to 74.5 (for UVLD) pmol min^{-1} tissue $equivalent^{-1}$. Qualitatively similar patterns were apparent between strains when activities were expressed relative to protein content of reaction mixtures (data not shown). Overall, MFO activities from these strains were poorly correlated ($r^2 = 0.46$) with susceptibilities to cypermethrin as measured with third-instar insects by Elzen *et al.* (28) following topical application of a dose corresponding to the LD_{80} for the susceptible, LSU strain (Fig. 1, inset).

Differences in frequency profiles for MFO activity from individual insects were measured between strains (Fig. 2). Activities in the LSU and RR-JU populations were distributed uniformly with greater than 90% of individuals expressing less than 30 pmol min^{-1} tissue $equivalent^{-1}$ (Fig. 2). In contrast, distributions of activities measured in samples of RR-AUG and UVLD insects were broad with more than 50% of the insects from these collections expressing levels of activity greater than 30 (and more than 20% with higher than 80) pmol min^{-1} tissue $equivalent^{-1}$.

Whereas larval MFO activities varied

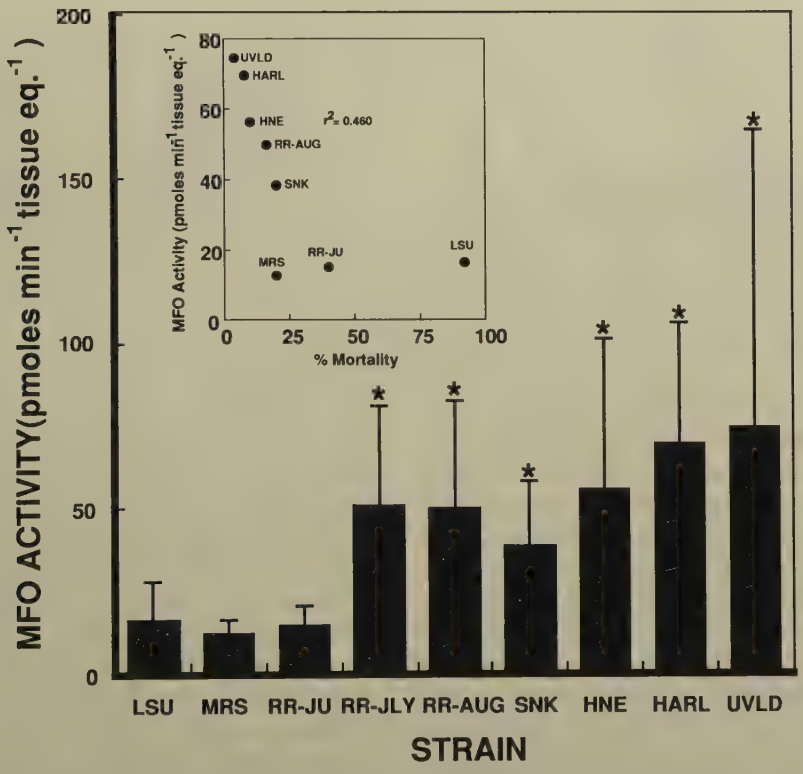

FIG. 1. *Midgut MFO activities measured from H. virescens larvae. Bars represent mean activity (expressed as pmol p-nitrophenol formed* min^{-1} *tissue* $equivalent^{-1} \pm SD$*) based on individual assays of 19–66 fifth-instar larvae. Asterisks denote activity that is significantly different from that measured for the LSU strain (Student's t test,* $\alpha = 0.01$*). Inset illustrates the relationship between MFO activity and mortality (measured in third-instar larvae for all strains except RR-JLY, 72 hr following topical application of 12.5 ng cypermethrin). Bioassay data are taken from Elzen et al. (25).*

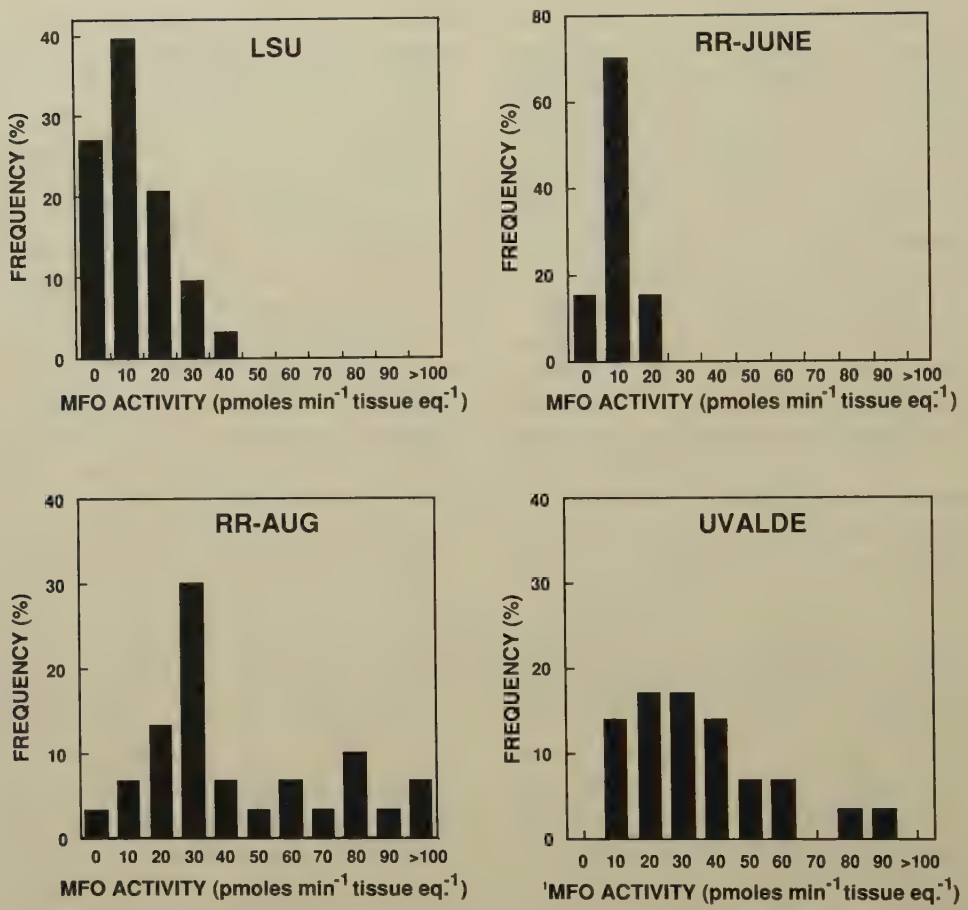

FIG. 2. *Frequency histograms for MFO activities (measured as pmol p-nitrophenol formed* min^{-1} $\pm$ *SD) from midguts of individual, fifth-instar larvae of H. virescens. Frequency is expressed relative to total numbers of larvae tested.*

with respect to the strain examined, levels of adult activity were relatively uniform and ranged from 56.5 (RR-JU) to 78.9 (HARL) pmol min^{-1} tissue $equivalent^{-1}$ (Fig. 3). In comparisons between life stages from the same strain, MFO activities (expressed per tissue equivalent) were higher in adults than in larvae. An opposite pattern was seen when activities were expressed relative to protein content in incubations (data not shown). However, regardless of the unit of activity used, there was a poor correlation ($r^2 < 0.20$, data not shown) between levels of activity expressed in larvae and adults from the same strain.

Larval GST activities toward CDNB and DCNB were measured in fat body and midgut homogenates from male and female insects of the susceptible LSU strain (Fig. 4). Activities measured toward both substrates in assays with female insects were consistently higher than those from male insects, but these differences were not statistically significant. In addition, no significant differences in larval GST activities toward either CDNB or DCNB (expressed per tissue equivalent) were apparent between homogenates of the two tissues examined. However, when activities were converted and expressed relative to protein content of incubations, GST activities toward CDNB (but not DCNB) were significantly higher in the midgut than the fat body (data not shown). Finally, regardless of the units of

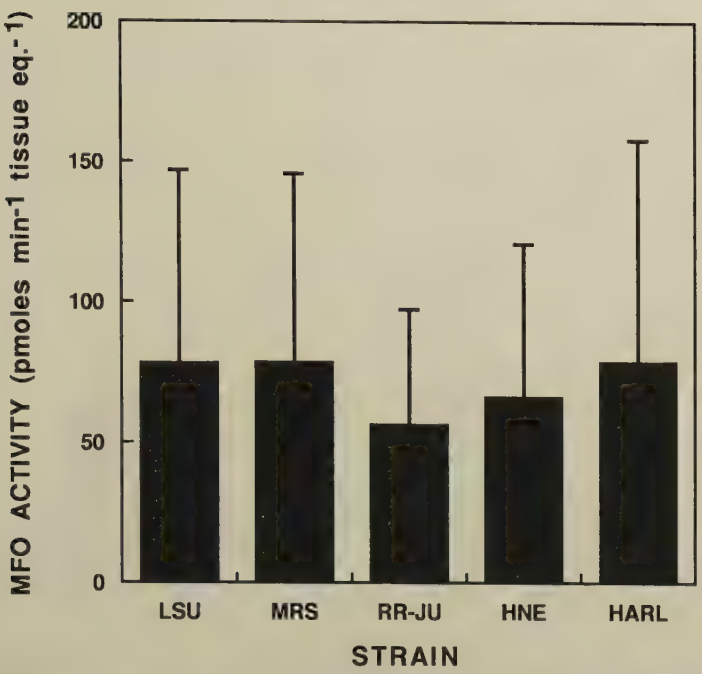

FIG. 3. *MFO activities measured from H. virescens adults. Bars represent mean activity (expressed as pmol p-nitrophenol formed min^{-1} tissue equivalent^{-1} ± SD) based on individual assays of 14–20 abdomens from 1-day-old, adult insects.*

activity, levels of GST activity toward CDNB were approximately 20- to 25-fold higher than those measured with DCNB in the LSU strain.

With the exception of insects from the HARL strain, field-collected larvae expressed GST activities toward both CDNB and DCNB that were higher than those measured from LSU insects (Fig. 5). GST activities were highest for CDNB in the SNK strain (106.3 nmol min^{-1} tissue equivalent^{-1}) and for DCNB in the UVLD strain (2.4 nmol min^{-1} tissue equivalent^{-1}). Lowest levels of activity with both substrates (13.4 and 0.61 nmol min^{-1} tissue equivalent^{-1} for CDNB and DCNB, respectively) were measured from HARL larvae. In general, activities with CDNB were about 20-fold higher than corresponding DCNB activities in these strains, with the exception of the SNK, HNE, and UVLD strains, where ratios of CDNB/DCNB activities were greater than 40. Similar patterns with respect to relative activity between strains and substrates were recorded regardless of whether activity was expressed per tissue equivalent or milligram protein. Larval GST activities toward CDNB were correlated ($r^2 = 0.834$; Fig. 5, inset) with those measured toward DCNB in these strains; however, poor correlations ($r^2 < 0.50$) existed between GST activities measured here (with either CDNB or DCNB) and levels of mortality measured by Elzen *et al.* (28) following topical application of a discriminating dose of cypermethrin (data not shown).

Frequency distributions of larval GST activities differed depending on the strain and substrate tested (Fig. 6). Distributions of larval activities toward DCNB were similar and the majority of individuals from the LSU, HARL, and HNE strains (64.7, 58.5, and 66.7%, respectively) expressed relatively low levels of activity (≤1.2 nmol min^{-1} tissue equivalent^{-1}; Fig. 6A). In contrast, distributions of larval GST/CDNB activities for field-collected strains were

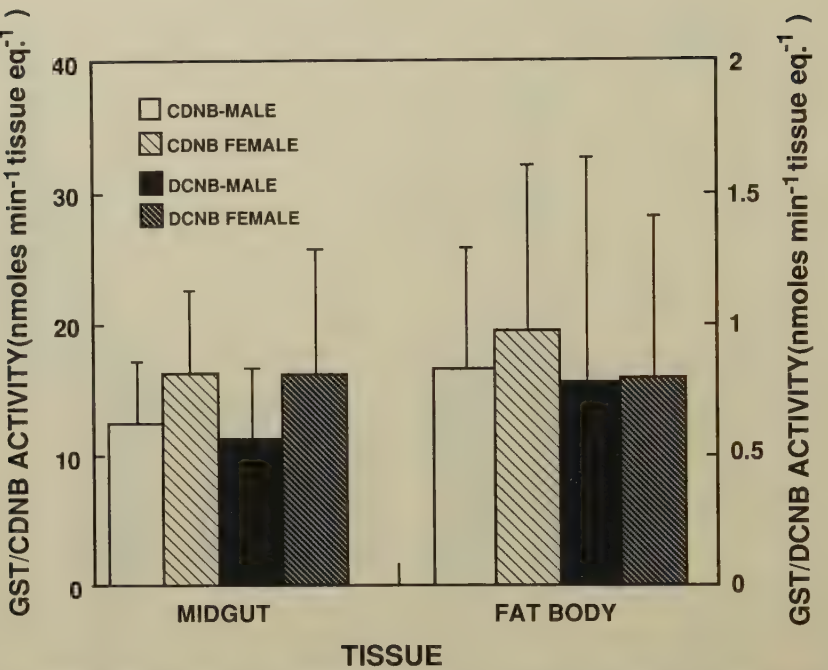

FIG. 4. *Midgut and fat body GST activities measured from male or female H. virescens larvae of the LSU strain. Bars represent mean activity (expressed as nmol substrate conjugated min^{-1} tissue equivalent^{-1} ± SD) based on individual assays of 30 fifth-instar larvae.*

broad and skewed toward higher levels of activity than those measured in the LSU strain (Fig. 6B). Whereas all individuals tested from the LSU strain expressed low levels of CDNB activity, the majority of insects from the HARL (79%) and HNE (67%) collections expressed activities ≥60 nmol min^{-1} tissue equivalent^{-1}.

Differences in levels and patterns of GST activities toward CDNB and DCNB were measured between larval and adult life stages of *H. virescens*. Mean levels of GST activities with DCNB in adults from field-collected colonies were not significantly different from those measured in the LSU strain (Fig. 7). In tests with CDNB, only HNE and HARL adults expressed activities that were significantly higher than those for LSU adults. In addition, ratios of CDNB/DCNB activities in adults were lower than those measured with larval insects. Adult GST activities toward CDNB were approximately 2-fold higher than corresponding DCNB activities for LSU, RR-JU, and MRS strains, and about 10-fold higher for HNE and HARL insects. Finally, a poor correlation (r^2 = 0.129) was found between CDNB and DCNB activities measured in these strains (Fig. 7, inset). Results of comparisons between strains with respect to levels of activity and CDNB/DCNB ratios were similar regardless of whether data were expressed relative to tissue equivalents or protein content in reaction mixtures; however, relative levels of activity between life stages differed depending on units of activity. Levels of GST activity (expressed per tissue equivalent) toward both CDNB and DCNB were consistently higher in adults than larvae, whereas larval activities were higher than those in adults (especially in tests with CDNB) when activity was expressed relative to protein content of assay incubations.

DISCUSSION

Insect MFOs and GSTs are believed to have major roles in insect–plant interac-

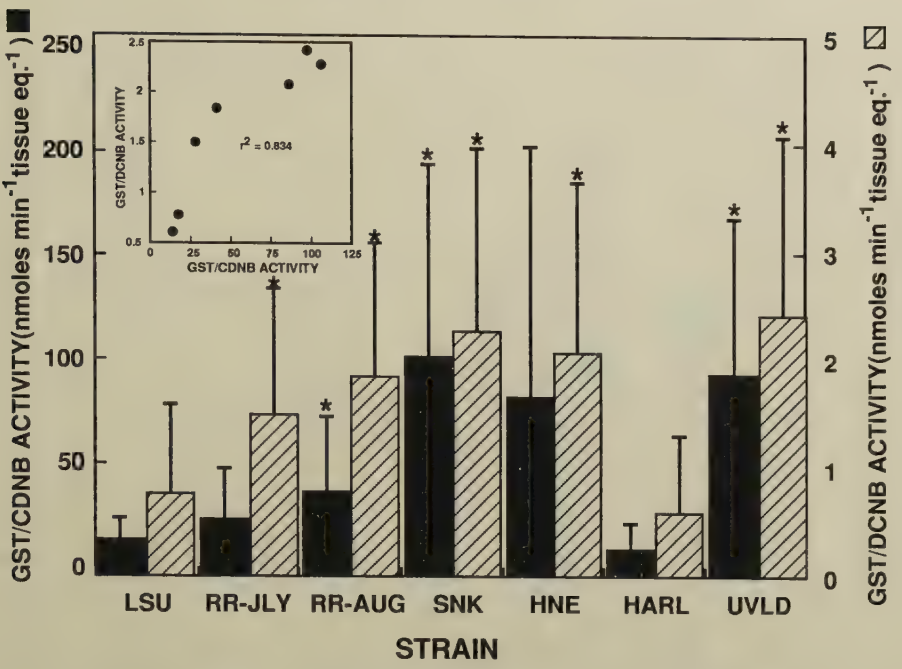

FIG. 5. *GST activities toward CDNB (solid bars) or DCNB (hatched bars) measured from H. virescens larvae. Bars represent mean activity (expressed as nmol conjugate formed* min^{-1} *tissue equivalent*$^{-1}$ ± *SD) based on individual assays of 25–40 fifth-instar larvae. Asterisks denote activity that is significantly different from that measured for the LSU strain (Student's t test,* $\alpha = 0.01$*). Inset illustrates the relationship between GST activities measured with CDNB and DCNB.*

tions (11, 21, 38–40) and in the development of resistance to insecticides (1, 41), including pyrethroids (26) and organophosphates (4, 42, 43). Conclusions regarding the ecological and toxicological significance of these enzymes are often drawn from studies using substrates that are neither allelochemical nor insecticidal; however, multiple forms of MFOs and GSTs are expressed in insects (2, 44) and the substrate specificities of these different forms are largely unknown. Further, temporal patterns of expression of these activities suggest a relationship with host plant feeding (21, 45), but the significance of developmental changes associated with the physiological roles of these enzymes (23, 46, 47) are rarely considered.

Through modifications of conventional assay protocols and the use of 96-well microplates, MFO and GST activities toward noninsecticide substrates were measured from tissues of individual tobacco budworms collected from cotton in agricultural areas where insecticides were providing less than adequate control (28, 48, 49). In general, larvae from field-collected strains were less susceptible to cypermethrin and most had higher levels of MFO activity than the LSU strain. However, three of the strains tested (LSU, RR-JU, and MRS) differed in their susceptibilities to cypermethrin but expressed similar levels of MFO activity. In addition, levels of susceptibility were similar between MRS and SNK collections despite statistically significant differences in mean levels of MFO activity between the two strains. Finally, preliminary results from studies measuring *in vivo* metabolism of cypermethrin indicate that, with the possible exception of the RR strains, enhanced metabolism is not a a major mechanism of resistance to this insecticide (50). In tests with the RR-AUG collection, cypermethrin susceptibility decreased more than twofold, and mean levels of me-

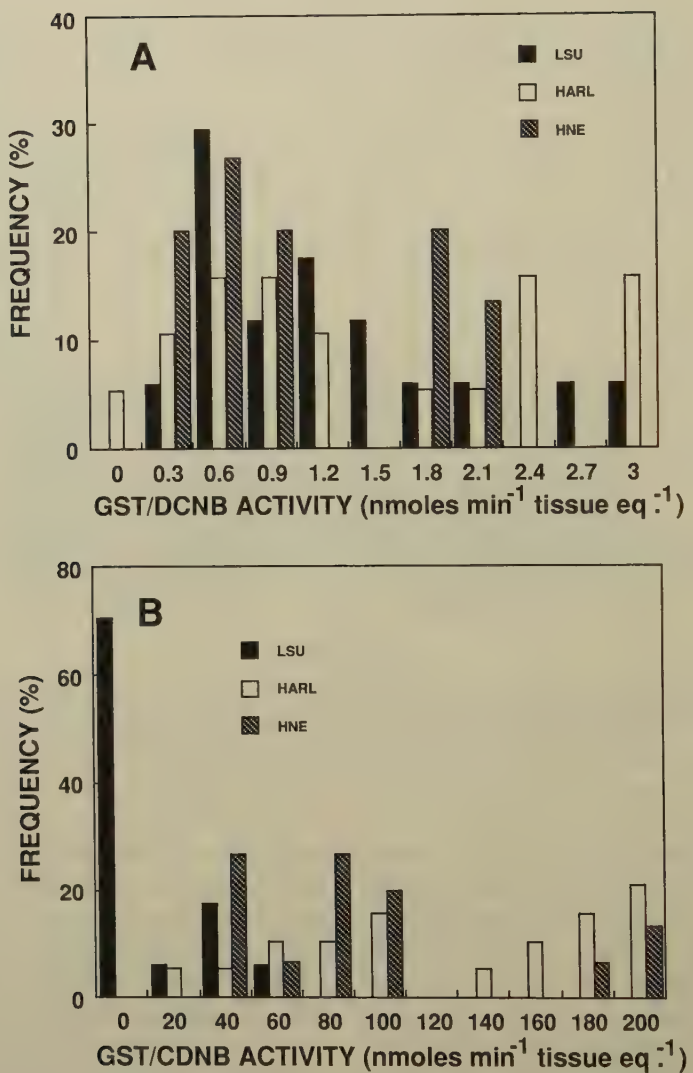

FIG. 6. *Frequency histograms for GST activities toward DCNB (A) or CDNB (B) from larval H. virescens. Strains tested include LSU (solid black bars), HARL (solid white bars), and HNE (hatched bars). Frequency is expressed relative to total numbers of larvae tested.*

tabolism of both PNA and cypermethrin were higher than in RR-JU insects, although differences measured in *in vivo* assays were not statistically significant between the two strains. These findings indicate that levels of detoxication enzyme activities measured toward surrogate (noninsecticide) substrates in field-collected strains of *H. virescens* may not be significant as indicators of cypermethrin metabolism and resistance.

Both MFO and GST activities were measured from tissue homogenates of adult insects and patterns of expression in adults were different from those in larvae. Mean levels of larval MFO activities were variable between strains and reflected differences in frequencies of individuals within these collections that expressed moderate to high levels of enzyme activity relative to the LSU strain. In contrast, there were no significant differences between strains in

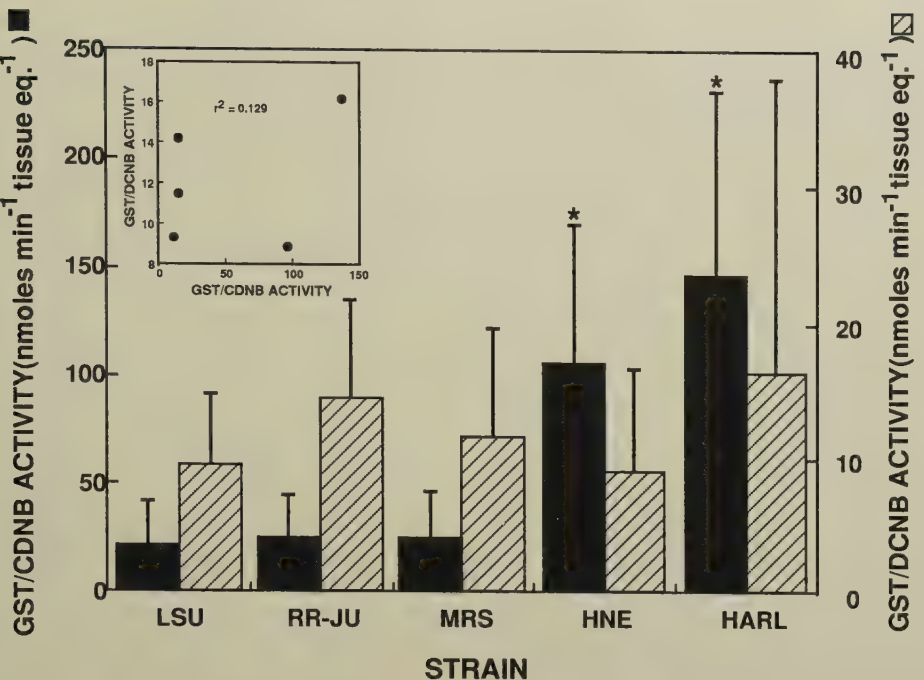

FIG. 7. *GST activities toward CDNB (solid bars) or DCNB (hatched bars) measured from H. virescens adults. Bars represent mean activity (expressed as nmol conjugate formed* min^{-1} *tissue equivalent*$^{-1}$ ± *SD) based on individual assays of 10–20 abdomens from 1-day-old adult insects. Asterisks denote activity that is significantly different from that measured for the LSU strain (Student's t test,* $\alpha = 0.01$*). Inset illustrates the relationship between GST activities measured with CDNB and DCNB.*

MFO activities measured in assays with adults. In tests with GSTs, mean levels of activity and patterns of substrate selectivity differed between the two developmental stages. Insects from the HARL strain expressed GST activities toward both CDNB and DCNB that were lowest in assays with larvae, but highest in assays with adults. In addition, larval GST activities measured from these strains toward CDNB were correlated with those measured with DCNB indicating that either the same enzyme(s) were responsible for the metabolism of the two substrates, or that enzymes metabolizing CDNB and DCNB were expressed coordinately in larval *H. virescens*. In contrast, there was no correlation between mean GST activities measured with the two substrates in adults. Finally, levels of MFO and GST activities expressed in larval insects were poorly correlated with those in adults. These results indicate that patterns of expression of detoxication enzymes may differ between larval and adult *H. virescens;* however, because there was no clear indication that the strains studied here express metabolic resistance, the implications of these findings with respect to insecticide metabolism and resistance are not known.

Our results also suggest that activities measured with surrogate substrates may be poor indicators of the contribution of MFO and GST activities in allelochemical metabolism and host plant utilization. In previous studies, variation in detoxication enzyme activities measured from insects at different developmental stages and the absence of detectable activity in adult lepidopteran insects have been attributed to differing levels of exposure to allelochemicals in the insect's diet (21, 45, 51, 52). As is the case for many holometabolous insects, there is little

or no overlap in feeding sites of immature and adult tobacco budworms: moths feed at various nectaries on cotton plants and larvae consume plant parts (mainly leaves and fruiting structures) that are known to contain a host of allelochemicals (reviewed in 53). Assuming that nectar is relatively toxin-free, the expression in adult insects of MFO and GST activities toward the substrates used here does not appear to be associated with the presence of plant toxins. Whereas adult MFO activity has been measured previously from a number of insect species (19, 20, 23, 54, 55) and has been shown to be associated with the metabolism of insecticides (56) and hormones (22), the role of these enzymes in adult *H. virescens* is not known.

In summary, although assays with surrogate substrates provide useful information regarding the expression of detoxication enzymes in insects, our data support the suggestion that exclusive use of these substrates does not necessarily provide insight that is of toxicological or ecological relevance (8, 11, 57). Further studies using insecticide substrates are necessary to determine the significance of qualitative differences in activities between developmental stages with respect to the potential for development of metabolic resistance to insecticides in this insect.

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Physiological Effects of Cornexistin on *Lemna pausicostata*

TADASHI AMAGASA,*,[1] REX N. PAUL,* JAMES J. HEITHOLT,† AND STEPHEN O. DUKE*,[2]

*USDA, *Agricultural Research Service, Southern Weed Science Laboratory and †Cotton Physiology and Genetics Research, P.O. Box 350, Stoneville, Mississippi 38776*

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Cornexistin, a microbial product isolated from the fungus *Paecilomyces variotii* SANK 21086, has postemergence herbicidal activity on several important weeds at rates (0.5 kg/ha) that do not affect maize (*Zea mays* L.). In a duckweed (*Lemna pausicostata* Hegelm. 6746) bioassay, cornexistin at 333 n*M* caused pronounced inhibition of growth within 72 hr. At the ultrastructural level, the first symptom was shrinkage and then disappearance of chloroplast starch grains, followed by plasma membrane and tonoplast disruption. Carbon dioxide fixation, as measured by infrared gas analysis, was reduced in a linear fashion by 10 μ*M* cornexistin, resulting in complete inhibition after 24 hr. Photosynthetic parameters more directly associated with light reactions, P-515 electrochromic transient and variable chlorophyll fluorescence, were more slowly affected by cornexistin. Five hundred micromolars each of L-aspartate, L-glutamate, or the TCA cycle intermediates, succinate, citrate, malate, oxaloacetate, and α-ketoglutarate prevented cornexistin phytotoxicity when these compounds were incorporated into the culture medium at the time of first exposure to the herbicide. D-Aspartate and D-glutamate had little or no effect on phytotoxicity. Sucrose at 15 m*M* was also very effective in reducing the phytotoxicity of cornexistin, whereas concentrations of 1 m*M* or lower were ineffective. Aminooxyacetic acid (AOA) at 3.33 μ*M* caused phytotoxicity effects similar to those of cornexistin. AOA effects were overcome by the same compounds that prevented cornexistin phytotoxicity. Cornexistin inhibited aspartate amino transferase (AAT) *in vitro* by only 20–30% at high concentrations, only after incubation in a cell extract. *In vitro* incubation of these *L. pausicostata* protein extracts also caused changes in AAT activity stain patterns on polyacrylamide gels. Cornexistin may be metabolized to an inhibitor of one or more AAT isozymes *in vivo*. © 1994 Academic Press, Inc.

INTRODUCTION

Phytotoxic microbially produced compounds have been of interest to those involved in herbicide discovery (1–4). One advantage of this strategy is that such phytotoxins often lead to molecular sites of action that have not been exploited by synthetic herbicides (1, 4). For example, the microbial product phosphinothricin is a potent inhibitor of glutamine synthetase (GS)[3] (5). Phosphinothricin (termed glufosinate when used as a herbicide) is the only successful commercial herbicide with GS as its target site (6). Scientific by-products of such research are new inhibitors that can be valuable tools in probing the physiology and biochemistry of plant enzymes.

Cornexistin (Fig. 1) is a nonadride (a compound with a nine-membered carbocyclic ring and one or more five-membered anhydride rings) compound isolated from a culture filtrate of the basidiomycete *Paecilomyces variotii* SANK 21086 (7). It has excellent herbicidal activity against both monocot and dicot weeds, but corn (*Zea mays* L.) is tolerant to it. Rubratoxin B, a related fungal metabolite, is only weakly phytotoxic (7). Nothing is known of the mechanism of action of cornexistin. In this paper, evidence is presented to support the view that cornexistin is an inhibitor of an

[1] On sabbatical from Sankyo Co., Ltd., Agroscience Research Laboratory, 1041 Yasu-cho, Yasu-gun, Shiga-ken 520-23, Japan.

[2] To whom correspondence should be addressed.-

[3] Abbreviations used: AAT, aspartate amino transferase; AOA, aminooxyacetic acid; CER, carbon dioxide exchange rate; GS, glutamine synthetase; PPFD, photosynthetic photon flux density; ppd, *p*-phenylenediamene.

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enzyme of primary carbon metabolism. Some of our data suggest that cornexistin may be a proherbicide that, in its active form, inhibits an aspartate amino transferase (AAT, EC 2.6.1.1; glutamate oxaloacetate transaminase when synthesizing L-Asp) isozyme. This is a site of action that has not been exploited by commercial herbicides.

MATERIALS AND METHODS

Plant material. Cultures of *Lemna pausicostata* Hegelm. 6746 were grown autotrophically in 5-liter plastic containers as before in a growth chamber under continuous illumination with white light (150 μmol m^{-2} sec^{-1} PPFD[4]) at 25°C (8, 9). Medium was renewed every 3 days. Under these conditions, doubling time of the colonies was approximately 2 days. Cucumber seedlings were grown as before (10) for P-515 electrochromic transient assays.

Phytotoxins and other test compounds. Cornexistin {[3S*, 4R*, 7R*]-3-*n*-propyl-4,7-dihydroxy-5-oxo-8(*Z*)-ethylidene-1-cyclononen-1,2-dicarboxylic acid anhydride} was purified from a culture filtrate of *P. variotii* SANK 21086 by the Sankyo Co., Ltd. fermentation research laboratories. The compound was extracted with organic solvents from culture filtrate, purified using column chromatography on Sephadex LH-20, and finally crystallized from methylene chloride (7). All other test compounds were obtained from Sigma Chemical Co.

Phytotoxicity assays. Fifteen colonies of three fronds each were selected from culture and placed in 6-cm-diameter polystyrene petri dishes with 6 ml of one-half strength Hutner's medium with or without cornexistin or other test compounds. Plants were exposed to continuous 150 μmol m^{-2} sec^{-1} PPFD at 25°C. Phytotoxicity was determined nondestructively by measurement of electrolyte leakage from the plants into the test media with a conductivity Meter (Model 4503A, Amber Science, San Diego, CA) (10, 11). Chlorophyll content was determined by the method of Hiscox and Israelstam (12).

Escherichia coli assays. The methods of Scheel and Casida (13) were adapted for these studies. *E. coli* cultures (provided by M. V. Subramanian of Sandoz Agro Inc.) were grown in minimal glucose medium (M9) with slight agitation at 37°C. Every 24 hr, 10 μl of old culture was used to inoculate 15 ml of fresh media in a 50-ml flask. To test the effects of cornexistin on bacterial growth, 150 μl of the above mature culture was used to inoculate 15 ml of minimal medium in 50-ml flasks. Cornexistin was dissolved in 15 μl of acetone for addition to the culture (final acetone conc. of 0.1%). The turbidity of 1-ml aliquots was determined spectrophotometrically at 580 nm against a blank containing only culture medium.

Enzyme assays. AAT extractions and assays were modifications of the methods of Duke *et al.* (14, 15). Enzyme preparations were made by homogenizing 3.5 g of *L. pausicostata* colonies with a ground glass homogenizer in a pH 8, 4°C extraction medium of 25 m*M* Tricine, 25 m*M* Hepes, and 2% (w/v) casein. The homogenate was filtered through four layers of cheesecloth and centrifuged at 500*g* for 3 min. The pellet was discarded and the supernatant used for enzyme assays. In some cases, the supernatant was centrifuged at 15,000*g* for 30 min. The supernatant of this centrifugation was used as one enzyme preparation and the mitochondria-containing pellet was resuspended in 3.3 ml of extraction medium. In some experiments, the low-molecular-weight compounds were removed from the enzyme extract by centrifugation of the extract at 3000*g* in centrifuge ultrafilters with a cutoff of 20,000 Da (Lide Manufacturing, Kenosha, WI).

The AAT assay reaction mixture consisted of 2.475 ml 0.1 *M* Tris–HCl (pH 7.5),

[4] Mention of a trademark or product does not constitute an endorsement of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

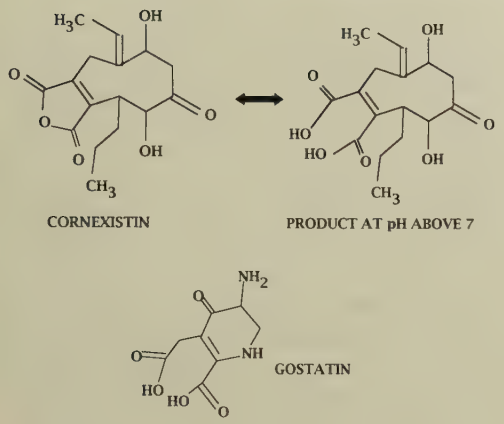

FIG. 1. *Structures of cornexistin, its hydrolysis product, and gostatin.*

0.2 ml 1 m*M* NADH, 0.1 ml of 0.33 *M* α-ketoglutarate, 0.1 ml of 0.33 *M* L-Asp, 1 unit of malate dehydrogenase (bovine heart, Sigma) in 0.01 ml, and 0.015 ml of cornexistin solution. The reaction was started by addition of 0.1 ml of enzyme preparation. Loss of NADH was spectrophotometrically monitored continuously at 25°C at 340 nm. The reaction was linear for up to 5 min, and assays were generally run for 3 min.

Malate dehydrogenase was assayed according to the method of Duke *et al.* (14).

Glutamate and malate pool sizes. L-Glu content of plants was measured by incubating 150 *L. pausicostata* colonies for 3 min in 3 ml of hot (84°C) water and then homogenizing in 2 ml of hot water. The homogenate was centrifuged at 15,000*g* for 30 min, the pellet was washed with 2 ml of hot water and centrifuged at 17,000*g* for 30 min, and the combined supernatants were lyophilized and stored at −20°C. Samples were dissolved in 1 ml of hydrazine buffer (0.4 *M* hydrazine, 0.5 *M* glycine-NaOH; pH 9.0). The assay consisted of the sample, 2.2 ml of hydrazine buffer, 0.2 m*M* NAD, and 2 units of Glu dehydrogenase in 0.005 ml. The reaction was run for 60 min at 30°C. Absorbance increase at 340 nm was used to quantify L-Glu content with L-Glu standards. Malate content of samples extracted as above was determined in reaction mixtures of 2.1 ml of hydrazine buffer (0.4 *M* hydrazine, 1 *M* glycine-NaOH; pH 9.5), 0.15 ml of 50 m*M* NAD, 0.75 ml of plant extract, and 2 units of malate dehydrogenase in 0.02 ml. The reaction was run at 30°C for 30 min, and malate was quantified by a standard curve, using increase in absorbance at 340 nm as an indicator of malate content.

Ultrastructure. Tissue was fixed and embedded with a modified procedure of Melaragno (16). Tissue was fixed in 0.4% glutaraldehyde in 10 m*M* cacodylate buffer (pH 7.0) for 1 hr. After washing in the buffer for 30 min, the tissue was postfixed for 30 min in 1% buffered OsO_4. During dehydration in acetone, at the 70% acetone step, the tissues were incubated in 1% *p*-phenylenediamine (ppd) in order to maximize lipid retention (17). The tissues were then rinsed in 70% acetone to remove unbound ppd and dehydration was continued. The tissues were embedded in Spurr's resin. Sections were made with a diamond knife, poststained with uranyl acetate and lead citrate, and observed and photographed with a Zeiss EM10 CR transmission electron microscope.

P-515 Electrochromic transient. The P-515 electrochromic transient was measured as described previously (18–20). Cotyledon discs (4 mm) were cut from 7-day-old cucumber seedlings and floated on aqueous solutions of cornexistin before assay. Fronds of cornexistin-treated *L. pausicostata* were mounted on sample holders with translucent tape, so that all light from the measuring and reference beams of the dual wavelength spectrophotometer that reached the photomultiplier tube passed through *L. pausicostata* fronds.

Infrared gas analysis. Carbon dioxide exchange rate (CER) of *L. pausicostata* cultures was measured with a LI-6200 portable photosynthesis system (LI-COR, Lincoln, NE). Before measurement, cultures containing 15 to 30 fronds were transferred to a 35-mm-diameter plastic petri dish containing deionized water. Dishes were then placed in a growth chamber at 25°C and a photosynthetic photon flux density (PPFD)

of 475 μmol $m^{-2}sec^{-1}$ for 30 min to allow for equilibration before the measurement. At the time of measurement, the petri dish lid was removed and the culture placed inside a 1-liter Plexiglas photosynthetic chamber that was mounted in the growth chamber. The CO_2 concentration was monitored for 2 min. Fresh weight of each culture was determined within 1 hr of the CER measurement and CER per unit fresh weight was calculated.

Variable chlorophyll fluorescence. Variable chlorophyll fluorescence was measured as before (21), except that two *L. pausicostata* colonies of three fronds each were arranged in the fluorescence detector probe so that they almost completely covered the probe surface. Similar to before (20), variable fluorescence was quantified as $(F_{max} - F_0)$-$(F_{terminal}/F_0)$. This function normalizes measurements of variable fluorescence.

Polyacrylamide gel electrophoresis. Plant tissue was homogenized in 25 m*M* Tricine/25 m*M* Hepes buffer (pH 8, 4°C) (*ca.* 1 g in 5 ml) with a ground glass homogenizer, filtered through four layers of cotton gauze, and centrifuged at 15,000*g* for 30 min. Protein in the supernatant was precipitated with five volumes of cold acetone by mixing and incubating at −20°C for 10 min. The precipitated protein was collected by centrifugation (10,000*g* for 5 min) and washed twice by repeated precipitation. After drying with a nitrogen gas stream, the protein was stored at −20°C.

The dried protein was dissolved in 25 m*M* Tricine, 25 m*M* Hepes (pH 8), loaded on a 10-cm tube (5-mm id) polyacrylamide gel (polyacrylamide content varied) with a 8-mm, 4% polyacrylamide stacking gel. Electrophoresis was with a running buffer of 25 m*M* Tris (pH 8.3) and 192 m*M* glycine run at 24 mA for 4 hr at 4°C.

In some experiments, the protein solutions or the gels were incubated for up to 3 hr in 0.1 *M* Tris–HCl (pH 7.5) with or without 1 m*M* cornexistin before electrophoresis or staining for AAT activity, respectively. ATT activity in the gel was determined according to Vallejos (22) by reacting the gel in the dark for 2 hr at 30°C in a reaction solution of 0.1 *M* Tris–HCl (pH 8.5), 7 m*M* α-ketoglutarate, 15 m*M* L-Asp, 0.4 m*M* pyridoxal-5-*P*, and 3.6 m*M* fast blue BB salt. With such a procedure, AAT activity appears as a blue band due to a reaction between AAT-generated oxaloacetate and fast blue BB. Ferguson plots of AAT activity stains were done according to Hames (23), using 5.5 to 8% polyacrylamide gels in 0.5% increments.

RESULTS

Toxicity to L. pausicostata and E. coli. Cornexistin at 3.3 μ*M* caused cellular leakage at 24 hr of exposure and at concentrations as low as 333 n*M* at 96 hr (Fig. 2A). At concentrations between 33 and 100 n*M* cornexistin-treated plants leaked less electrolyte than did the untreated control plants. At low cornexistin concentrations (33 and and 100 n*M*), the conductivity of the bathing solution was reduced. There was no effect of cornexistin on electrolyte leakage in darkness over the same time periods with these concentrations of the phytotoxin (data not shown). There was no growth of the colonies in the dark, with or without cornexistin (data not shown). The dose–response relationships for growth inhibition were similar to those for cellular leakage, whereas there was relatively little effect on chlorophyll content (Fig. 2B). Concentrations of cornexistin between 3.33 and 100 n*M* stimulated growth of the plants as much as 35%. The I_{50} values for the maximal effect of cornexistin on electrolyte leakage decreased logarithmically with time (Fig. 3). The I_{50} values for inhibition of growth were similar to those for electrolyte leakage at the same time points (Fig. 3). There was no effect of cornexistin at concentrations up to 1 m*M* on the growth of *E. coli* during 25 hr of exposure, during which the culture doubled approximately eight times (data not shown).

Ultrastructural effects. A range of ultra-

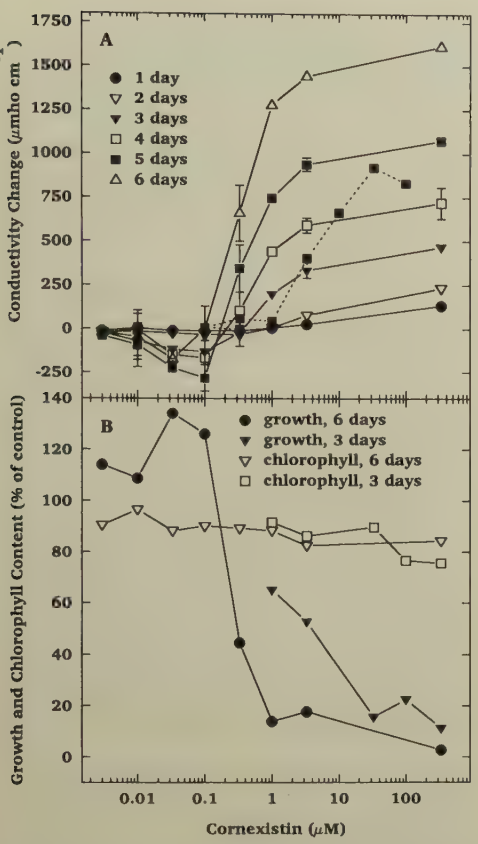

FIG. 2. *Dose–response for cellular leakage (A), as determined by change in conductivity of growth medium, and fresh weight and chlorophyll content (B) of L. pausicostata as affected by exposure to cornexistin. The dotted line in (A) is the effect of AOA on cellular leakage at 5 days. Error bars are ±1 SE of the mean of three dishes of colonies.*

structural effects of cornexistin could be found between cells in a single plant at various times after exposure; however, there was a trend toward total cellular disintegration with time. Chloroplasts of control tissues contained large starch grains after 24 hr of continuous light (Fig. 4A) and throughout the 120-hr time course. Incubation of tissues for the same time period in 333 n*M* cornexistin reduced the size of the starch grains (Fig. 4B). After 48 hr, there was no change in the untreated controls; however, the chloroplasts of treated tissue contained fewer and much smaller starch grains, disorganized thylakoids, and sometimes small vesicles (Figs. 4C and 4D). After 72 hr, all starch was depleted in cells of treated plants (Fig. 5A). Chloroplasts of cornexistin-treated plants sometimes contained large osmiophilic bodies, and the tonoplast and/or plasma membranes of these cells was sometimes broken. By 120 hr of treatment, only vestiges of thylakoids were left in some cells of treated plants, whereas, earlier stages of cellular disintegration were found in less damaged cells (Fig. 5B).

Effects on photosynthesis. Carbon dioxide fixation was rapidly reduced by both 3.3 and 10 μ*M* cornexistin (Fig. 6A). The effect of the higher concentration was linear from first exposure until 100% inhibition after 24 hr.

Effects of cornexistin on photosynthetic parameters more closely linked to light reactions appeared more slowly than the effects on carbon dioxide fixation. Variable fluoresence was reduced slowly over a 2-day period (Fig. 6B). There was no change in the shape of the variable fluorescence transient other than that caused by a reduction of F_{max} (data not shown). Over 48 hr of continuous light there was a decrease in variable fluorescence in the control tissues. However, the reduction was greater

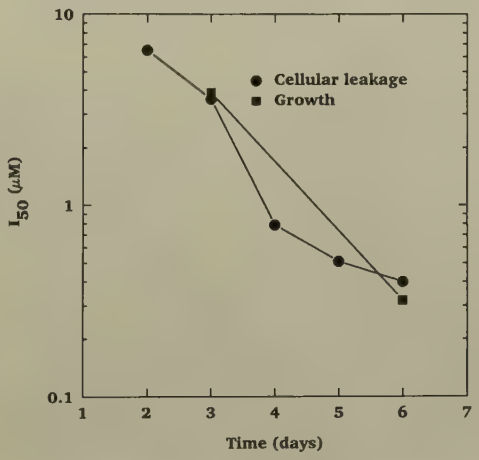

FIG. 3. *Cornexistin I_{50} values (concentration required for 50% inhibition) for effects at different times after treatment of L. pausicostata.*

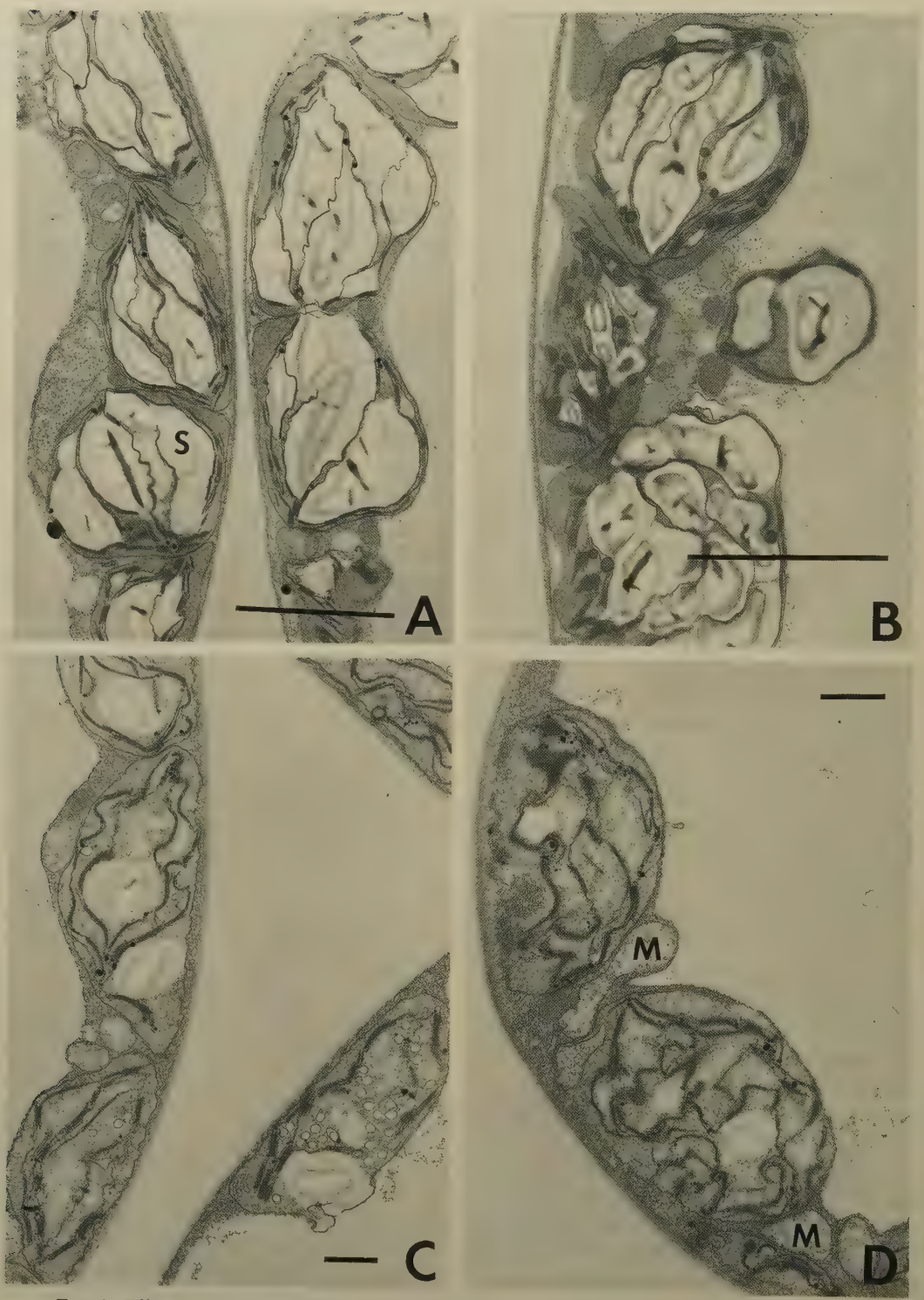

FIG. 4. *Effects of 333 nM cornexistin on ultrastructure of L. pausicostata mesophyll leaf cells. (A) Untreated cells after 24 hr. Bar = 5 μm. (B) Treated cells after 24 hr. Bar = 5 μm. (C and D) Treated cells after 48 hr treatment. S, starch grain; M, mitochondria. Bars = 1 μm.*

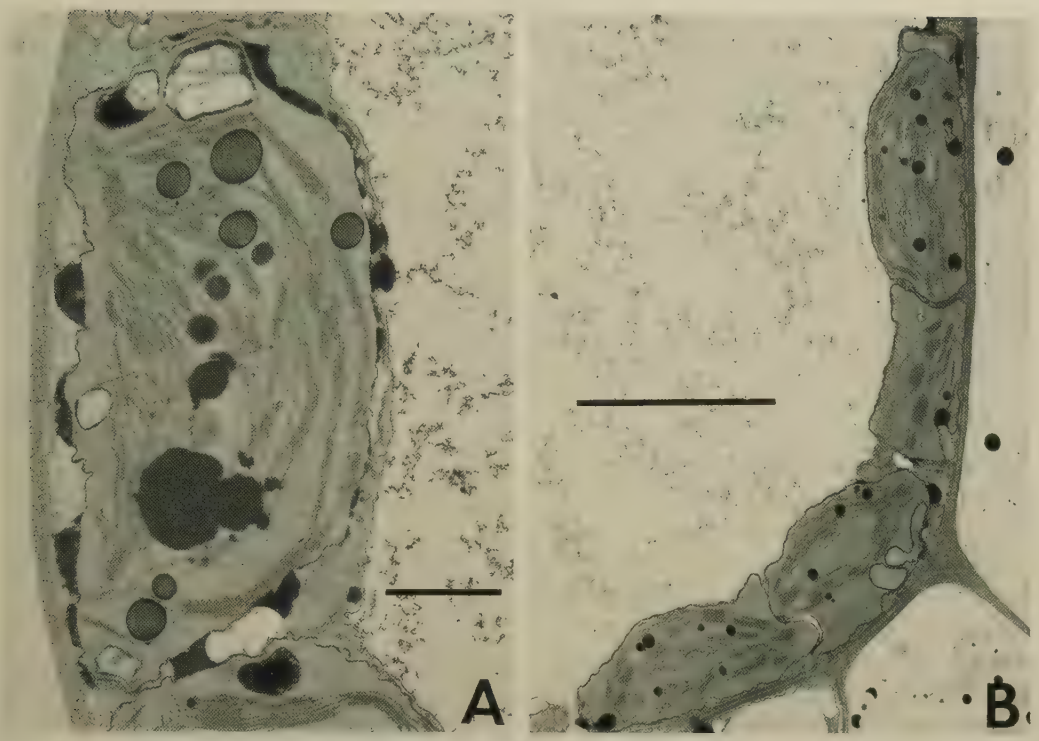

FIG. 5. *Effects of 333 nM cornexistin on ultrastructure of L. pausicostata mesophyll leaf cells. (A) Treated cell after 72 hr.* (B) Treated cell after 120 hr treatment. *(A) Bar = 1 μm; (B) bar = 5 μm.*

in the cornexistin-treated tissues, so that there was a significant reduction in both 3.3 and 10 μ*M* treatments by this time. Even at 48 hr, however, variable chlorophyll fluorescence was not completely suppressed by 10 μ*M* cornexistin.

The P-515 electrochromic transient is a measure of the proton gradient created across thylakoid membranes by the Hill reaction (20). The larger the amplitude of the electrochromic transient, the greater the proton gradient. It is directly affected by CF_1 ATPase inhibitors, protonophores, and other compounds that interfere with this

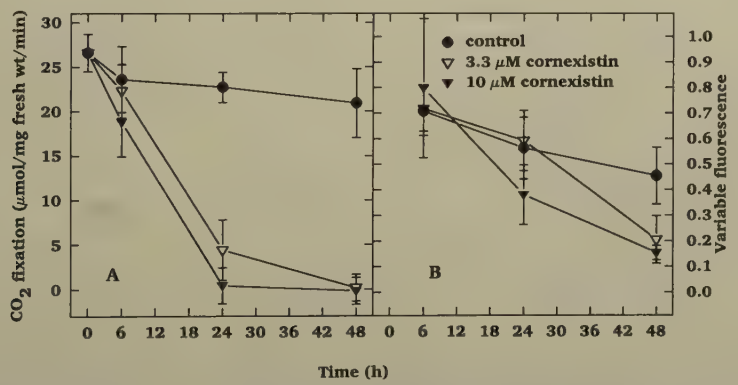

FIG. 6. *Effects of cornexistin on carbon dioxide fixation (A) and variable chlorophyll fluorescence (B) of L. pausicostata colonies over a 48-hr time course under continuous illumination. Error bars are ±1 SE of the mean.*

gradient. Cornexistin reduced the P-515 electrochromic transient response in a log dose-dependent manner in *L. pausicostata* and cucumber cotyledon discs after 24 and 51 hr exposure, respectively (Fig. 7). The effect of cornexistin on this parameter appeared to be saturated at about one-third of the control value in both cucumber and *L. pausicostata*.

Effects of glycolytic intermediates, sucrose, and amino acids. The relatively slow development of phytotoxicity and lack of dramatic phytotoxic symptoms is somewhat like the symptomatology of some herbicides that inhibit amino acid synthesis. In some cases, the effects of amino acid synthesis inhibitors can be overcome by supplying affected tissues with exogenous amino acids at the time the plants are exposed to the inhibitor. In initial attempts to ameliorate the phytotoxic effects of cornexistin with L-amino acids, the L-amino acids Ala, Leu, Val, Ile, Glu, Ser, Phe, Asp, Gln, Arg, Orn, Cys, Met, His, Asn, Thr, and Lys, each at 80 μ*M*, and Tyr, Trp, and Met, each 40 μ*M*, were supplied together to plant colonies with 1 μ*M* cornexistin. The solution of 20 amino acids had no effect on electrolyte leakage by itself, and it reduced

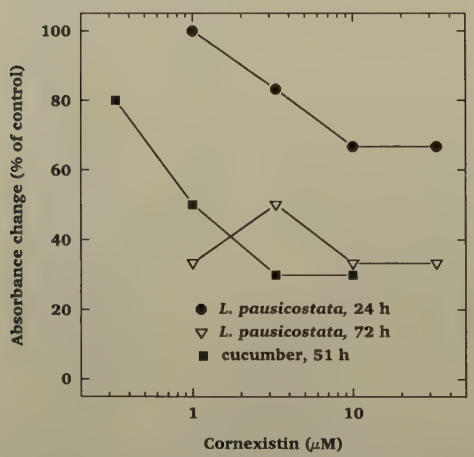

FIG. 7. *Effects of various cornexistin concentrations on the P-515 electrochromic transient in L. pausicostata and cucumber cotyledon discs. Values are averages from 5 to 10 measurements. The control values for L. pausicostata and cucumber were 0.003 and 0.005* $A_{518-490\ nm}$, *respectively.*

TABLE 1

Effects of Amino Acids on 1 μM Cornexistin-Caused Cellular Leakage from L. pausicostata Plants after 4 Days Treatment

Amino acids	Effect (% reversal)
200 μ*M* Phe + 40 μ*M* each Tyr and Trp	4.3
200 μ*M* Gln	12.8
200 μ*M* Arg	11.5
200 μ*M* Cys + 40 μ*M* Met	−5.2
200 μ*M* His	−2.5
200 μ*M* Pro	−23.1
200 μ*M* Asn	7.4
200 μ*M* each Asp, Glu, and Ala	32.5
200 μ*M* each Lys and Thr plus 40 μ*M* Met	−12.3
200 μ*M* each Leu, Val, and Ile	−3.8
500 μ*M* Ala	−5.1
500 μ*M* Asp	49.3
500 μ*M* Glu	36.1

the effect of the phytotoxin by about 20% (data not shown). When supplied singly or in mixtures of related amino acids, L-Glu and L-Asp were most effective in reducing cornexistin-caused cellular leakage (Table I). The effects of L-Asp and L-Glu on the herbicidal effects of 333 n*M* cornexistin were pronounced at 3, 4, and 5 days after the beginning of exposure, with virtually complete reversal of the effects at 1 m*M* of either amino acid (Fig. 8). At 0.33 m*M* each

TABLE 2

Concentrations of Amino Acids and Organic Acids Required to Reduce the Phytotoxicity of 333 nM Cornexistin to L. pausicostata as Determined by Electrolyte Leakage 4 and 5 Days after Treatment

Amino or organic acid	Conc. needed for 50% recovery (μ*M*)	
	4 Days	5 Days
Asp	530	505
Glu	580	530
Citrate	205	150
Succinate	260	190
Malate	670	350
Oxaloacetate	250	190
Acetate	360	—
Pyruvate	380	280
α-Ketoglutarate	205	180

of the two amino acids, they acted synergistically in alleviating the phytotoxicity of cornexistin (Fig. 8). D-Glu and D-Asp had no effect on the phytotoxicity of cornexistin (data not shown).

Tricarboxylic acid cycle intermediates also reduced or eliminated the phytotoxicity of cornexistin. For example, oxaloacetate, pyruvate, and acetate at 1 m*M* completely alleviated the phytotoxicity of 333 nM cornexistin at 4 days (Fig. 9). A summary of the effects of amino and organic acid effects on cornexistin phytoxicity is provided in Table 2. Citrate, succinate, and

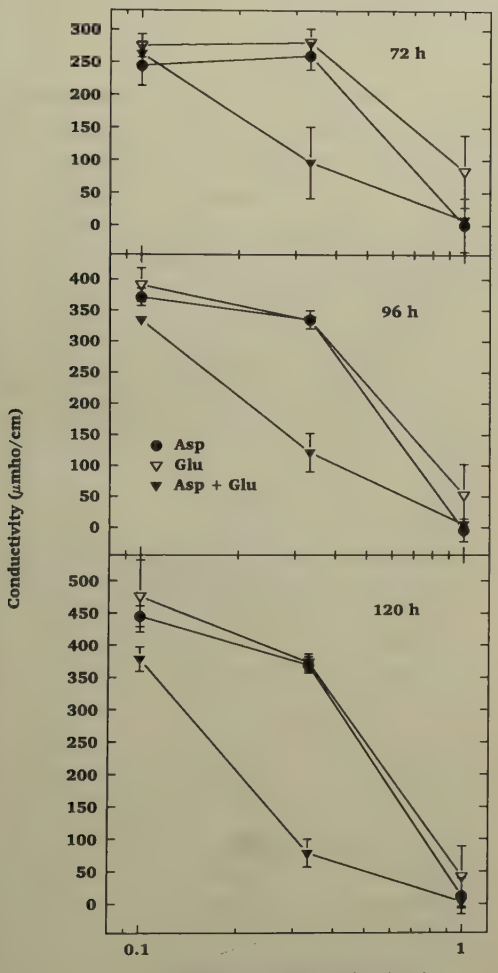

FIG. 8. *Effects of different concentrations of Asp and Glu (log scale) on phytotoxicity of 333 nM cornexistin to L. pausicostata after various periods of exposure. Error bars are ±1 SE of the mean.*

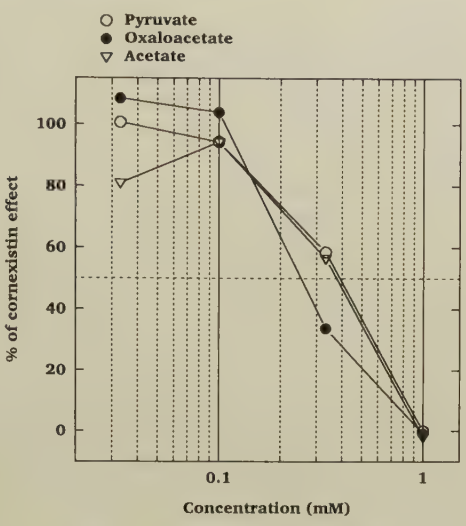

FIG. 9. *Effects of different concentrations of TCA cycle intermediates on phytotoxicity of 333 nM cornexistin to L. pausicostata after 4 days.*

α-ketoglutarate, each provided singly, were most effective in eliminating the phytotoxicity of 333 nM cornexistin.

Sucrose at 44 m*M* (1.5%, w/w) was effective in reducing the phytotoxicity of a range of cornexistin concentrations (Fig. 10A). At 14.6 m*M*, sucrose partially reversed the effect of 3.3 μ*M* cornexistin (Fig. 10B). At concentrations of 0.1, 0.33, and 1 m*M*, sucrose has little or no effect on phytotoxicity of cornexistin (data not shown).

We thought that the low concentrations of cornexistin needed for an effect were not consistent with inhibition of RuBisCo, an enzyme found in great abundance. However, we hypothesized that the effect could be due to inhibition of an enzyme involved in carbon dioxide fixation present at levels that would more closely match the low concentration of the inhibitor required for an effect. Carbonic anhydrase is a potential candidate. Acetazolimide is an inhibitor of this enzyme (24). Dose–response studies with acetazolimide indicated that a concentration of about 1 to 10 m*M* had phytotoxic effects similar to 3.3 μ*M* cornexistin (data not shown). Neither L-Asp nor citrate feeding had any effect on the phytotoxicity of acetazolimide (data not shown).

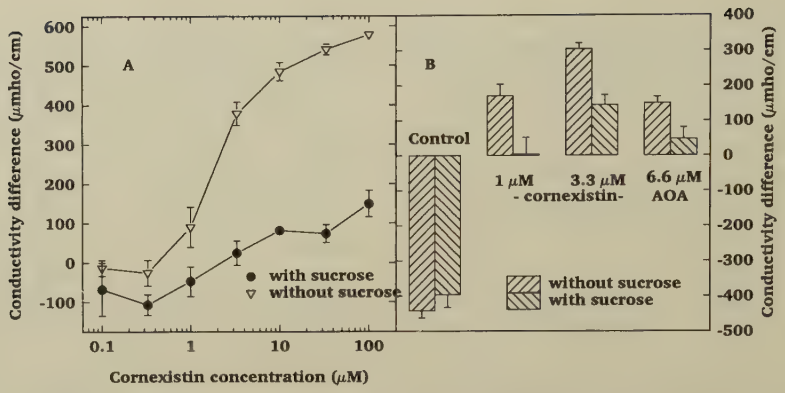

FIG. 10. *Effect of sucrose on cornexistin effects on electrolyte leakage of intact L. pausicostata plants. (A) Effects of 44 mM (1.5%, w/v) sucrose on effects of various concentrations of cornexistin on cellular leakage after 4 days. (B) Effects of 14.6 mM (0.5%, w/v) sucrose on electrolyte leakage caused by either cornexistin or AOA after 4 days. Error bars are ±1 SE of the mean.*

Comparison of AOA to cornexistin. AOA is an inhibitor of AAT, an enzyme involved in Asp and Glu metabolism. The phytotoxic symptomatology caused by AOA was similar to that of cornexistin. Cornexistin was slightly more effective as a herbicide than was AOA after 5 days of exposure (Fig. 2A). Furthermore, the same amino acids, glycolytic intermediates, and sucrose that alleviated the plants of phytotoxic effects of cornexistin were also effective for AOA (Figs. 10B & 11).

In vitro and in vivo effects on AAT. L. pausicostata extracts contained easily measurable AAT activity that was strongly inhibited by AOA; however, cornexistin had no effect on its activity, even at relatively high concentrations (Table 3). The same result was obtained with concentrations of L-Asp and α-ketoglutarate from 0.33 to 11 m*M* in the assay mixture (data not shown). We also found no effect on malate dehydrogenase (data not shown). Cornexistin is easily converted to its hydrolysis product (Fig. 1) at basic pH. Such a conversion could occur *in vivo*. We converted cornexistin to its hydrolysis product *in vitro* and

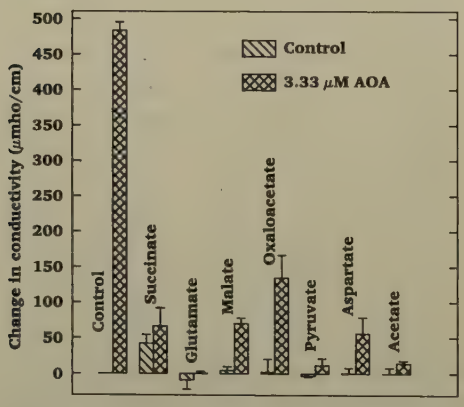

FIG. 11. *Effects of Asp, Glu, and TCA cycle intermediates on phytotoxicity of AOA after 96 hr of treatment of L. pausicostata. Error bars are ±1 SE of the mean.*

TABLE 3
Effects of Cornexistin and AOA on AAT Activity Extracted from L. pausicostata

Inhibitor (μ*M*)	Activity (μmol/min/g fresh wt)	% inhibition
	Cytoplasmic extract	
None	0.413 ± 0.023	0
Cornexistin (10)	0.399 ± 0.008	3.5
(100)	0.416 ± 0.008	−1.0
	Mitochondrial fraction	
None	0.099 ± 0.021	0
Cornexistin (10)	0.077 ± 0.012	21.8
(100)	0.092 ± 0.021	7.4
	Hydrolyzed cornexistin	
None	0.159	0
Hydrolyzed cornexistin (200)	0.141	12.3
(670)	0.148	6.9

tested its effects on AAT. It had no inhibitory activity (Table 3). However, when cornexistin was incubated in a *L. pausicostata* extract for 1 to 6 hr at 25°C, the amended extract consistently inhibited about 25 to 30% of the AAT activity (Table 4, Fig. 12A). The pH of the incubation and enzyme assay media (pH 6.5 to 8) had little influence on the effectiveness of cornexistin as an AAT inhibitor (Fig. 12B). The difference between the activity of AAT with and without cornexistin after incubation of cornexistin for 5 hr in a cell-free extract was consistently 20 to 30% from pH 6.5 to 8. The amount of casein in the extraction medium had no influence on this effect (data not shown). Cornexistin up to 2 m*M* had no inhibitory action on AAT activity of extract which had been incubated without cornexistin with L-Asp and α-ketoglutarate concentrations from 0.33 to 11 m*M*. Furthermore, cornexistin at concentrations up to 2 m*M* had no effect on malate dehydrogenase with oxaloacetate concentrations from 1.33 to 13.3 m*M*.

AOA is a slow-binding, irreversible inhibitor of AAT (25). Treatment of plants with AOA for 1 or 2 days led to strong reductions in extractable AAT activity, however, the same concentrations of cornexistin resulted in relatively small reduction in extractable activity (Table 5). The AOA-treated plants appeared to be recovering from treatment after 2 days; whereas, the effect of cornexistin on reduction of extractable activity increased from 1 to 2 days.

Increasing the ratio of plant extract added to the AAT activity assay reduced the specific activity of untreated plants, but only slightly reduced that from plants treated with cornexistin (Table 6). This effect could be explained by the presence of a higher level of either an inhibitor or a competing enzyme in the control extract than that of the cornexistin-treated plants. Boiled crude extract had little effect on activity of active crude extract, suggesting that the inhibitor or interfering factor is heat labile (data not shown). Removing lower-molecular-weight compounds (<20,000 Da) by centrifugation of the extract through centrifuge ultrafilters eliminated the inhibiting effect of high extract concentrations on AAT activity in the extracts of control plants (Table 6).

However, plant extracts incubated with or without 1 m*M* cornexistin for 5 hr were unaffected by the low-molecular-weight fraction (data not shown). Furthermore, the AAT activity of the plant extracts treated *in vitro* with cornexistin was not recovered by separation of low-molecular-weight compounds by filtrate centrifugation (data not shown).

Cornexistin effects on metabolite levels. Cornexistin at 3.3 μ*M* for 48 hr had no effect on L-Glu levels in *L. pausicostata* colonies. We found 2.26 and 2.31 μmol per gram of fresh weight in control and treated colonies after 48 hr. Similarly, we found no effect of either 3.3 or 10 μ*M* cornexistin on malate content of tissues after 48 hr treatment.

Cornexistin effects on AAT isozyme patterns. There are several isozymes of AAT found in plant cells associated with different cellular compartments, and AAT isozymes are sometimes found within the same compartment (26). Activity stains of native PAGE disc gels of extracts of control and cornexistin-treated plants had several bands of activity, ranging from about 2.2 to 7.8 R_f values, but there was no difference in the AAT activity band patterns between treated and untreated plants (not shown). No AAT activity was detected when D-Asp was substituted for L-Asp (not shown). However, when extracts of proteins from untreated plants were incubated for several hours (2 to 5 hr) in 1 m*M* cornexistin, obvious changes in migration of AAT activity on the discs gels could be detected (Fig. 13). This effect was best visualized in 6% gels. This incubation resulted in an approximately 25% reduction in AAT activity in test tube assays, as described above. Treatment with cornexistin resulted in loss or re-

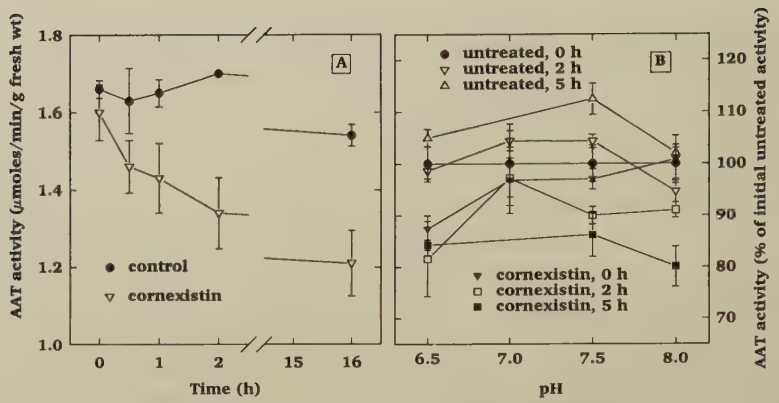

FIG. 12. *(A) Time course for generation of an AAT inhibitor during incubation of cornexistin in cell-free extract of L. pausicostata. An AAT-containing extract of L. pausicostata was incubated at 25°C for the times indicated with or without 1 mM cornexistin, after which AAT activity was measured. Between 2 and 16 hr (overnight) the extracts were incubated at 3°C. Error bars are 1 SE of the mean. (B) Effects of pH of incubation and assay media on effectiveness of cornexistin as an AAT inhibitor. Error bars are ±1 SE of the mean.*

duction of the lowest R_f bands and the appearance of two new, higher R_f bands. Ferguson plots resulted indicated that the molecular mass of the three highest R_f bands of the cornexistin treatment were the same (not shown). Thus, they varied mostly in electrical charge.

Incubation of the gels in cell-free extract with or without cornexistin before activity staining prevented visualization of AAT activity because of interference by a component(s) of the cell-free extract. Incubation of the protein preparations with a protease inhibitor solution (1 m*M* phenazine methosulfate, 10 μ*M* pepstatin, 10 μ*M* leupeptin, and 5 m*M* 1,10-phenanthroline) had no influence on the cornexistin effect, supporting the Ferguson plot indication that there was no difference in molecular mass of the isozymes.

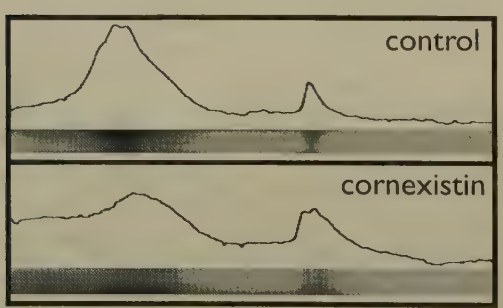

FIG. 13. *AAT activity stains on 6% polyacrylamide disc gels after electrophoresis. L. pausicostata protein preparations were incubated with or without 1 mM cornexistin before electrophoresis and staining.*

DISCUSSION

The ultrastructural effects of cornexistin are unlike those previously described for any herbicide (6, 27). PS II electron transport inhibitors cause rapid loss of starch grains, but they also concomitantly cause thylakoid disruption (*e.g.*, 21, 27, 28). In these studies, starch grains disappeared rapidly, but the chloroplast was the last organelle to lose its integrity. Carbon dioxide fixation was rapidly inhibited by cornexistin, and this effect did not appear to be due to effects on the light reactions of photosynthesis. Even at the longest incubation periods at the highest cornexistin concentrations, there was little or no loss of chlorophyll (Fig. 2B). The ultrastructural symptomatology after starch grains disappeared was similar to that caused by protoporphyrinogen oxidase-inhibiting herbicides (10, 29), but developed at a much slower rate.

The site of action of cornexistin is not likely to be an enzyme found in great abundance, such as RuBisCo, because of the effectiveness of very low concentrations of

TABLE 4
AAT Activity of Extract from L. pausicostata Plants Incubated with Cornexistin or AOA for Various Time Periods before the Assay Was Run

Treatment	Incubation time (hr) 0	1	2	5.5
	AAT activity [nmol/min/g fresh wt (% inhibition)]			
Untreated	1.43	1.40	1.46	1.38
Incubated in 1 m*M* cornexistin before assay		1.06 (25)	1.04 (29)	1.03 (25)
1 m*M* cornexistin added after incubation			1.47 (0)	1.37 (1)
Incubated in 0.05 m*M* AOA before assay			0.088 (94)	
0.05 m*M* AOA added after incubation			0.185 (87)	

cornexistin. Herbicidal RuBisCo inhibitors have been discovered, but none are commerical herbicides (6). I_{50} values were submicromolar after 3 days of exposure. Few herbicides are this active. The decreasing I_{50} values with time (Fig. 3) suggest that cornexistin is a prophytotoxin; that is, a compound that is metabolized to a phytotoxin within the plant.

The feeding experiments show that the phytotoxicity caused by AOA, a potent AAT inhibitor, and cornexistin have similar patterns of the compounds that ameliorate their activity. However, feeding studies can sometimes be misleading. For example, TCA cycle intermediates will ameliorate the effects of glyphosate in some cases (30), even though the target site of this herbicide is enolphosphoshikimate phosphate synthase, a shikimate pathway enzyme (31). In this case, the effect was explained by the fact that inhibition of EPSP synthase deregulates the shikimate pathway, resulting in uncontrolled carbon flow from the glycolytic pathway to the shikimate pathway.

TABLE 5
AAT Activity Extracted from L. pausicostata Plants Treated with AOA and Cornexistin

Inhibitor (μ*M*)		μmol/min/g fresh wt (% inhibition) 24 hr	48 hr
None		0.700 (0)	0.550 (0)
Cornexistin	(0.33)	0.664 (5.1)	0.473 (14)
	(3.33)	0.664 (5.1)	0.486 (11.6)
AOA	(3.3)	0.193 (72.4)	0.276 (49.8)
	(33)	0.070 (90.0)	0.161 (70.7)

The cells became starved for these intermediates before they were depleted of the aromatic amino acid end products of the shikimate pathway.

Although sucrose reduced the phytotoxicity of cornexistin, it was much less effec-

TABLE 6
Effects of Amount of Plant Extract on AAT Activity in Assays of Extracts from L. pausicostata Grown With or Without 10 μM Cornexistin for 48 hr

Treatment	Extract volume[a] (ml)	AAT activity (μmol/min/g fresh weight)[b]
	Experiment 1	
Control	0.2	1.72 ± 0.10
	0.4	1.51 ± 0.08
	0.8	0.55 ± 0.21
Cornexistin	0.2	1.89 ± 0.06
	0.4	1.86 ± 0.04
	0.8	1.64 ± 0.13
	Experiment 2[c]	
Control		
Unfiltered	0.2	0.94
Filtered	0.2	0.78
Unfiltered	0.8	0.45
Filtered	0.8	1.11
Cornexistin		
Unfiltered	0.2	0.63
Filtered	0.2	0.49
Unfiltered	0.8	1.18
Filtered	0.8	1.27

[a] Extract volume used in a total AAT activity assay volume of 3 ml.

[b] ±2 SE of the mean.

[c] Compounds of molecular mass less than 20,000 Da were either removed by centrifuge filtration (filtered) or not (unfiltered).

tive than tricarboxylic acids, L-Asp, or L-Glu. This finding indicates that the effect of cornexistin is not a direct effect on photoassimilate production.

AOA is a well-known inhibitor of AAT, and was demonstrated here to strongly inhibit AAT of *L. pausicostata*. Yet, native cornexistin has no effect on AAT *in vitro*. Furthermore, extraction of relatively high AAT activities from cornexistin-treated plants indicates that, if AAT is inhibited *in vivo* by a modified product of cornexistin, the inhibition is reversible. There was a strong reduction in the number and size of starch grains by 2 days; whereas the effect on extractable AAT activity was negligible at this time. At high extracted protein concentrations *in vitro*, as might be expected *in vivo*, the total AAT activity was sometimes actually higher in cornexistin-treated plants (Table 6).

Gostatin (Fig. 1) is a *Streptomyces sumanensis* metabolite that was reported to irreversibly inhibit AAT (32). One end of the gostatin molecule is a structural analog of the hydrolysis reduction product of cornexistin (Fig. 1). Therefore, it is possible that this product of cornexistin is metabolized to an AAT-inhibiting analog of gostatin. If so, however, the high levels of extractable AAT activity indicate that the inhibition is reversible. Although Nishino and Murao (32) stated that gostatin is an irreversible inhibitor, there were no data in their paper to support this claim.

That cornexistin had no effect on *E. coli* growth could be due to either of two reasons. *E. coli* may not contain the site of action that the phytotoxin targets in plants. This could be due to lack of the target enzyme entirely or to a modified binding site. The alternative is that both organisms contain the binding site, but only plants have the capacity to activate cornexistin to the proper inhibitory form. AAT exists in both *E. coli* and plants. Therefore, *E. coli* may not have the necessary enzyme(s) to activate cornexistin if AAT is the site of action.

Proherbicides and prophytotoxins are known, but are not common (33). Examples are the esters of aryloxyphenoxy propanoate herbicides that must be deesterified in order to inhibit acetyl CoA carboxylase (34) and metabolic conversion of methazole to the PS II inhibitor *N*-methyl-*N'*-dichlorophenylurea (35). The microbial product bialaphos is metabolically converted by plants to the potent GS inhibitor, phosphinothricin (36). Most known AAT inhibitors contain an amino or aminooxy moiety (37). Thus, it is possible that cornexistin is aminated *in vivo* in order to be activated.

The consistent 20 to 30% inhibition of AAT by preincubated cornexistin suggests that it may inhibit one subclass or isozyme of AAT. The PAGE data (Fig. 13) support this. Incubation of *L. pausicostata* extracts with cornexistin eliminated some isoforms and caused the appearance of others. Whether the activity of one isoform or more was totally inhibited or whether its activity was only reduced and its mobility on the gel changed is unclear.

The cornexistin analog rubratoxin B is an inhibitor of (Na^+ and K^+)-ATPase (I_{50} = 6 μM) of animals (38). An indicator of CF_1 ATPase activity of the chloroplast is the P-515 electrochromic transient (18–20). Cornexistin reduced the P-515 signal, but the effect was not complete, nor was it highly sensitive, compared to other parameters. CF_1 ATPase inhibitors, such as tentoxin, inhibit dissipation of the proton gradient generated by photosystem II (20). A reduction in the signal could be due to a loss of photosystem II activity, increased membrane permeability to protons, or an increase in CF_1 ATPase activity. Regardless, the effect on the P-515 signal did not appear to be sufficient to be a causal factor in phytotoxicity.

Several approaches to further research on the mechanism of action of this toxin are suggested from our results. A cytochemical approach at the transmission electron microscope level might allow determination of whether one AAT isozyme or more are af-

fected by cornexistin *in vivo*. Such experiments could also reveal the cellular site of inhibited isozymes. Using the method of Paul (39), we have been unsuccessful with this approach in *L. pausicostata* because of low *in vivo* AAT activity and the fragility of the cells. Another approach is to fractionate the cells and to repeat some of the experiments described in this paper on fractions enriched in specific AAT isozymes. A third approach would be to determine the degradation products of cornexistin in a susceptible plant species and to determine the *in vitro* activity of these compounds on AAT and other enzymes. We expect that such further studies will confirm that the mechanism of action of cornexistin is unique.

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Effect of Fenpiclonil on Macromolecule Biosynthesis in *Fusarium sulphureum*

AD B. K. JESPERS[1] AND MAARTEN A. DE WAARD

Department of Phytopathology, Wageningen Agricultural University, P.O. Box 8025, 6700 EE Wageningen, The Netherlands

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Fenpiclonil is a phenylpyrrole fungicide used for control of *Fusarium sulphureum*. Under growth-limiting glucose concentrations, the fungicide (0.4 μ*M*) specifically inhibited incorporation of glucose and mannose into fungal macromolecules. This was not observed with a less fenpiclonil-sensitive laboratory isolate of *F. sulphureum*. Toxicity of phenylpyrrole analogues ranked with their inhibitory effect on mannose incorporation. The highest inhibitory effect was found for incorporation of glucose into hyphal wall glycans. Biosynthesis of these glycans is mediated by glycan synthetases in the plasma membrane. However, these enzymes are probably not the target site of fenpiclonil, since fenpiclonil neither inhibited glycan synthetase activity in cell-free extracts nor caused accumulation of uridinediphosphoglucose in mycelium. Fenpiclonil also inhibited incorporation of ^{14}C-labeled glucose into other fungal macromolecules such as proteins. These results suggest that fenpiclonil does not interfere with various polymerization steps in the biosynthesis of macromolecules, but with an initial metabolization step of glucose such as glucose phosphorylation. This hypothesis would also explain the inhibitory effect of fenpiclonil on incorporation of [^{14}C]uracil into uridine and UDP-activated sugars. © 1994 Academic Press, Inc.

INTRODUCTION

Fenpiclonil is the first phenylpyrrole fungicide developed by Ciba-Geigy (AG, Basel; Fig. 1 (1)). The fungicide, at concentrations which reduced growth by about 50% (1 μ*M*), instantaneously inhibits accumulation of substrates such as amino acids and monosaccharides in *Fusarium sulphureum* (2). This observation suggests that fungitoxicity may be a consequence of a yet unknown primary effect of the fungicide on membrane function (2). In the present study, the effect of fenpiclonil (0.4 μ*M*) on accumulation and incorporation of various substrates into macromolecules of *F. sulphureum* at growth-limiting glucose concentrations was studied. Similar experiments were carried out with a less fenpiclonil-sensitive isolate. Various phenylpyrrole analogues were tested as well. The site of inhibition in the various biosynthetic processes was determined by isolation and identification of mycelial fractions containing different types of macromolecules, especially those containing various types of polysaccharides.

MATERIALS AND METHODS

Organism, Culture Methods, and Sample Preparation

The *F. sulphureum* isolate 1743 was obtained from the fungal collection of the Research Institute for Plant Protection (IPO) in Wageningen. The less fenpiclonil-sensitive isolate 1743.1 originated from a fast-growing sector in a culture of isolate 1743 on potato dextrose agar (PDA)[2] amended with fenpiclonil (4 μ*M*). Reduced sensitivity to fenpiclonil correlates with high sensitivity to osmotic stress and loss of

[1] Present address: Household and Personal Care Research B.V., P.O. Box 16299, 2500 AE The Hague, The Netherlands.

[2] Abbreviations used: low M_r, water-soluble metabolites of low molecular weight; PAW, phenol/acetic acid/water extract; PDA, potato dextrose agar; SLS, sodium lauryl sulfate; TCA, trichloroacetic acid; TLC, thin-layer chromatography, UDPGlc, uridine diphosphoglucose; UDPGlc-NAc, uridine diphospho-*N*-acetylglucosamine.

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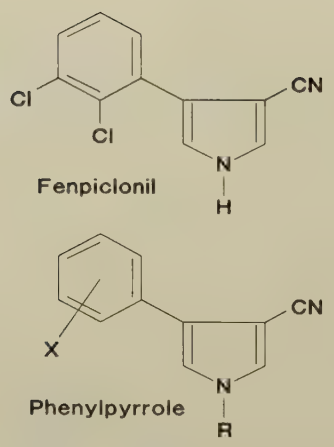

FIG. 1. *Structure of fenpiclonil and general structure of phenylpyrrole fungicides.*

pathogenicity (2). The isolates were subcultured weekly on PDA at 20°C. Czapek Dox liquid cultures were inoculated with macroconidia, which were obtained from 2-week-old cultures on PDA, to a final concentration of 2×10^5 conidia ml^{-1}. Cultures were incubated in a rotary shaker at 120 rpm and 20°C for 2 days. Mycelium was harvested and washed with demineralized water by vacuum filtration over a filter paper in a Büchner funnel. Standard mycelial suspensions were obtained by resuspending the washed mycelium in Czapek Dox liquid medium without glucose. Before the experiments, suspensions were allowed to settle for 1 hr. In all experiments, mycelial suspensions were treated by preincubation with fenpiclonil from 200-fold stock solutions in methanol for 15 min. Methanol (0.5%) served as control. All experiments were performed at least two times in duplicate in a rotary shaker at 120 rpm and 20°C.

Chemicals

Czapek Dox liquid medium was purchased from Oxoid Ltd. (London, UK). PDA and TLC plates (silica gel 60 F_{254} and PEI-cellulose F_{254}) were obtained from Merck (Darmstadt, FRG). Radiolabeled *N*-acetyl-[1-^{14}C]glucosamine (sp act 2.0 GBq $mmol^{-1}$), L-[U-^{14}C]aspartic acid (sp act 7.88 GBq $mmol^{-1}$), [1-^{14}C]glucose (sp act 2.1 GBq $mmol^{-1}$), [6-^{14}C]glucose (sp act 2.0 GBq $mmol^{-1}$), D-[U-^{14}C]glucose (sp act 10.8 GBq $mmol^{-1}$), D-[U-^{14}C]mannose (sp act 9.3 GBq $mmol^{-1}$), D-[2-^{3}H]mannose (sp act 570 GBq $mmol^{-1}$), [*methyl*-^{3}H]thymine (sp act 1.74 GBq $mmol^{-1}$), [2-^{14}C]uracil (sp act 1.92 GBq $mmol^{-1}$), and uridine diphospho-D-[U-^{14}C]glucose ammonium salt (sp act 12.0 GBq $mmol^{-1}$) were obtained from Amersham International Ltd. (Amersham, UK). Aqualuma was purchased from Lumac BV (Schaesberg, The Netherlands). β-1,3-Glucanase from *Helix pomatia* was obtained from Fluka (Buchs, Switzerland). Technically pure fenpiclonil (98.49%, CGA 142705) and the phenylpyrrole analogues CGA 106877, CGA 106884, CGA 117106, CGA 129973, CGA 152908, CGA 153322, CGA 173506, and CGA 266258 were gifts of Ciba-Geigy AG (Basel, Switzerland). All other chemicals were of technical grade.

Accumulation and Incorporation Studies

Substrates were added to standard mycelial suspensions (10 ml) from 200-fold-concentrated stock solutions in water. Final concentrations were 0.0017, 10, 25, 200, 200, and 200 μ*M* for [*methyl*-^{3}H]thymine, [2-^{14}C]uracil, [U-^{14}C]aspartic acid, [U-^{14}C]mannose, [1-^{14}C]glucose, and [6-^{14}C]glucose, respectively. Simultaneously with radiolabeled thymine, uracil, and aspartic acid, cold glucose to a final concentration of 200 μ*M* was added. Incubation was stopped after 15 min. Accumulation into mycelium and subsequent incorporation of substrates into macromolecules were assessed as described previously (2, 3).

The ratio of accumulated and incorporated mannose and aspartic acid in mycelium of *F. sulphureum* isolates 1743 or 1743.1 was determined in single Erlenmeyer flasks. The substrate mixture consisted of glucose, [2-^{3}H]mannose, and [U-^{14}C]aspartic acid at final concentrations of 100, 25, and 25 μ*M*, respectively. The experiment was performed as described

previously (2). Mycelium was hydrolyzed in 0.6 *N* perchloric acid (1 ml) at 90°C for 30 min and suspended in 10 ml Aqualuma Plus (3). Radioactivity of both isotopes was determined with a Beckman LS6000 TA scintillation counter in the dual-channel mode (windows 0–350 and 350–670) after 15 hr. The system was calibrated with quenched ^{3}H and ^{14}C standards (4).

Fractionation of Mycelium

Incorporation of mannose into (glyco)-proteins was assessed in standard mycelial suspensions (10 ml) incubated with 100 μ*M* D-glucose and [U-^{14}C]mannose (5000 Bq ml^{-1}) for 15 min. After incubation, mycelium was collected on Whatman GF/A filters, washed with ice-cold demineralized water, and resuspended in ice-cold water (0.5 ml). Subsequently, ice-cold $CHCl_3$/CH_3OH (1/1, 3 ml) was added within 30 sec (5, 6). This procedure yielded homogeneously dispersed mycelium and further mechanical disruption was not necessary. Mycelial residues were washed twice by centrifugation with $CHCl_3/CH_3OH/H_2O$ (1/1/0.3, 3 ml). All extracts were combined and water-soluble metabolites of low molecular weight (low M_r) were obtained from the upper layer after phase separation with water (3 ml).

Water-soluble and cell wall proteins were extracted three times from mycelial residues with phenol/acetic acid/water (2/1/1, 2 ml) in a shaking water bath at 70°C for 30 min (PAW extraction (7, 8)). The combined extraction fluids were mixed with 300 μl ammonium formate (10%) and 100 μl ovalbumin (50 mg ml^{-1}). (Glyco)proteins were precipitated by addition of acetone (35 ml) at −20°C for 1 hr and hydrolyzed in 1 *N* NaOH and 1 *N* $NaBH_4$ at 100°C for 1 hr (9). Oligosaccharide side chains were separated from proteins by passing the mixture over anion-exchange columns (0.5 × 2 cm, AG 1-X8, 100–200 mesh). The columns were equilibrated and saccharides eluted with the hydrolyzation medium.

Incorporation of glucose into fungal macromolecules was assessed in standard mycelial suspensions incubated in 100 μ*M* D-[U-^{14}C]glucose (10 ml, 7500 Bq ml^{-1}) for 15 min. The incubation was stopped and metabolites (low M_r) were extracted according to the procedure described above. Water-soluble macromolecules (glycoprotein, protein, DNA, RNA, etc.) were extracted with 3 ml sodium lauryl sulfate (SLS, 1%) in 50 m*M* Tris–HCl, pH 8.0, at 0°C for 1 hr (10). Mycelial residues (hyphal walls) were subsequently fractionated in two steps with 1 *N* NaOH (3 ml) at 60°C for 20 min (fraction I), with 0.5 *N* HCl (3 ml) at 100°C for 1 hr (fraction II), and with 1 *N* NaOH (3 ml) at 60°C for 20 min (fraction III (11)). Between changes of extraction fluid, mycelial residues were washed with demineralized water (3 ml). Wash water was discarded. The extracts were neutralized and (glyco)proteins were extracted with equal volumes of phenol saturated with 10 m*M* acetic acid. The water phase contained primarily glycans.

The size of the extracted glycoprotein-attached oligosaccharides and cell wall glycan fragments was estimated by Bio-Gel-P4 gel filtration chromatography (range from 1 to 12 sugar residues). The column (1.0 × 100 cm) was eluted with 50 m*M* Tris–HCl, pH 7.5, 50 m*M* NaCl, and 0.02% NaN_3 at a flow rate of 8 ml hr^{-1} (12). Extracted glycans were hydrolyzed to monomers by complete acid hydrolysis in 6 *N* H_2SO_4 at 120°C for 3 hr (13). The nature of the glycan monomers was determined by paper chromatography using ethanol/pyridine/water (8/2/1) as solvent system (14). Digestion of β-1,3-glucan was performed with purified β-1,3-glucanase (0.1 mg ml^{-1}) from *H. pomatia* in 25 m*M* Tris–Cl, pH 7.0, at 25°C for 24 hr (10).

Uracil Metabolites

Standard mycelial suspensions were incubated as described for the glucose metabolism experiments, except for the presence of [2-^{14}C]uracil (200 Bq ml^{-1}) in the sub-

strate mixture. Uracil metabolites were extracted from incubated mycelium with 50% ethanol (5 ml) at 0°C for 1 hr and dried in an Eleya rotary vacuum evaporator (15). Uracil metabolites were separated by thin-layer ion-exchange chromatography on PEI-cellulose plates using 0.25 *M* LiCl as solvent system (16). Radioactive spots were located by autoradiography using Kodak X-AR films and identified by R_f value using authentic standards.

Cell-Free Glycan Biosynthesis

Washed mycelium was suspended in FAGE buffer (25 m*M* Hepes, pH 7.4, 0.1 *M* glycerol, 10 m*M* NaF, 1 m*M* α-toluenesulfonyl fluoride, 5 m*M* EGTA, and 1% bovine serum albumin fraction V) at a density of 0.1 g wet wt ml^{-1} and disrupted four times at 0°C for 30 sec with 30-sec intervals in a Bead-Beater (Biospec Products, Bartlesville, OK (17)). β-1,3-Glucan synthetase activity in the supernatant fraction (500*g*, 5 min) was determined by the method of Quigley and Selitrennikoff (18, 19). Assay mixtures in FAGE contained protein and UDPGlc at final concentrations of 2 mg ml^{-1} and 100 μ*M*, respectively.

RESULTS

Accumulation and Incorporation

The effect of fenpiclonil (0.4 μ*M*) on accumulation of substrates into mycelium and incorporation into macromolecules was studied in liquid cultures at growth-limiting glucose concentrations. Under similar conditions with normal glucose concentration, the fungicide inhibited mycelial growth by 15% (2). The substrate concentrations used allowed linear accumulation during the experiment. Accumulation levels never exceeded 50%. Inhibition of accumulation of the various substrates ranged from 7 to 28% (Table 1). The fungicide did not inhibit incorporation of the accumulated substrates thymine, uracil, aspartic acid, and *N*-acetylglucosamine into DNA, RNA, protein, and chitin, respectively (Table 1). In contrast, incorporation of glucose and especially of mannose into fungal macromolecules was significantly inhibited (Table 1).

In a following experiment, effects of various concentrations of fenpiclonil on accumulation and incorporation of [2-^{3}H]mannose and [U-^{14}C]aspartic acid were tested (Fig. 2). The accumulation of mannose and aspartic acid in untreated mycelium of iso-

TABLE 1

Effect of Fenpiclonil (0.4 μM) on Accumulation of Radiolabeled Substrates by Fusarium sulphureum *Isolate 1743 and Incorporation of the Radiolabel into Macromolecules during 15 min of Incubation*

Substrate	Concentration (μ*M*)	Accumulation[a]		Incorporation[a]	
		Control (%)[c]	Fenpiclonil[b] (%)	Control (%)[d]	Fenpiclonil[b] (%)[d]
[*methyl*-^{3}H]Thymine[f]	—[e]	0.16	0.12 (78)	33.1	33.5 (101)
[2-^{14}C]Uracil[f]	10	4.5	4.2 (93)	5.3	5.6 (106)
[U-^{14}C]Aspartic acid[f]	25	26.7	24.3 (91)	7.9	8.4 (106)
N-Acetyl-[1-^{14}C]glucosamine[f]	10	41.8	31.5 (75)	70.6	83.3 (118)
[U-^{14}C]Mannose	200	2.7	2.2 (83)	26.8	14.2 (52)
[1-^{14}C]Glucose	200	20.4	14.7 (72)	14.6	8.8 (60)
[6-^{14}C]Glucose	200	20.0	14.4 (72)	28.4	18.2 (64)

[a] Mean results of two experiments. Variation in repetitions less than 5%.
[b] Fenpiclonil added 15 min prior to addition of substrates.
[c] Accumulation in mycelium as percentage of label added.
[d] Incorporation in macromolecules as percentage of label accumulated.
[e] [*methyl*-^{3}H]Thymine was added at the highest specific activity available (final concentration 1.7 n*M*).
[f] Medium amended with cold glucose (200 μ*M*).

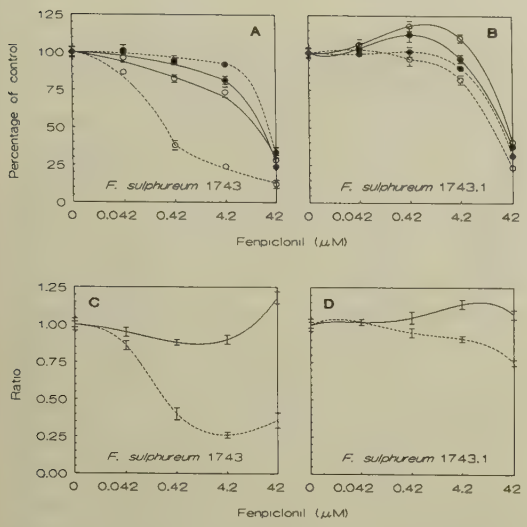

FIG. 2. *Effect of fenpiclonil on accumulation (—) and incorporation (---) of [U-^{14}C]aspartic acid (25 μM, ●) and [2-^{3}H]mannose (25 μM, ○) in Fusarium sulphureum isolates 1743 and 1743.1 in a medium containing cold glucose (100 μM). Figures are expressed as percentage relative to the untreated control (top panels). The bottom panels represent the ratio between accumulated mannose and accumulated aspartic acid (—) and between incorporated mannose and incorporated aspartic acid (---) for isolate 1743 and 1743.1, respectively. Data are mean values of three experiments carried out in duplicate.*

late 1743 was 1.4 and 37% and in untreated mycelium of isolate 1743.1 1.3 and 30% of the label added, respectively. The values for incorporation of mannose and aspartic acid into fungal macromolecules were 0.54 and 1.6% for isolate 1743 and 0.56 and 1.8% for isolate 1743.1, respectively. Fenpiclonil inhibited accumulation of mannose and aspartic acid in isolate 1743 to about the same degree (Fig. 2A). In contrast, incorporation of mannose into fungal macromolecules was inhibited more strongly than incorporation of aspartic acid. The concentration of fenpiclonil which reduced the incorporation ratio by 50% was 0.1 μ*M* (Fig. 2C). Almost no inhibitory effects on these parameters of the less-sensitive isolate 1743.1 were observed (Figs. 2B and 2D).

Various analogues differing from fenpiclonil in fungitoxicity were tested in the same system. Their inhibitory effect on mannose incorporation ranked positively with toxicity (Table 2).

Effect on Composition of Fungal Macromolecules

When mycelium was allowed to accumulate [U-^{14}C]mannose for 15 min, the major part of radioactivity taken up (65%) was recovered in CO_2 released from the culture medium (Table 3). Accumulation of the label in the $CHCl_3/CH_3OH$ fraction, containing the various biosynthetic intermediates, increased upon fenpiclonil treatment. A minor part (10.8%) of the accumulated label in mannose was incorporated into macromolecules. Since mannose in mycelium is mainly present in the oligosaccharide side chains of glycoproteins (20), the PAW fraction was isolated. Fenpiclonil (0.4 μ*M*) appeared to inhibit incorporation of the label in mannose into this fraction by 50%. Incorporation of the label in mannose into other mycelial macromolecules (residue) was inhibited by 56%.

Strong acid hydrolysis of the total macromolecule fraction (PAW extract and residue) yielded sugar monomers. The major part (>70%) was identified as glucose and a minor part as mannose (results not shown).

The effect of fenpiclonil (0.4 μ*M*) on incorporation of the label in [U-^{14}C]glucose into macromolecules was also studied. In this experiment, the main components of the mycelial macromolecular fractions were analyzed by sequential extraction and digestion (11) (Table 4). The SLS extract contained water-soluble glycans, protein, RNA and DNA (14), fraction I (first NaOH digest) proteins, and long-chain β-1,3-glucans (>12 sugar residues, β-1,3-glucanase digestion yielded exclusively sugar monomers; results not shown). The predominant part of fraction II (HCl digest) contained short-chain glycans. Glycans in fraction III (second NaOH digest) consisted of long-chain glycans (probably complex β-1,3-1,6-glucans) and monosaccharides. The remaining fraction was chitin (11).

TABLE 2

Effect of Various Fenpiclonil Analogues on Radial Growth (EC_{50}), Accumulation of [2-^{3}H]Mannose (25 μM), and [U-^{14}C]Aspartic Acid (25 μM) in Mycelium and Incorporation of the Radiolabel into Macromolecules of Fusarium sulphureum *Isolate 1743 in a Medium Containing Cold Glucose (100 μM)*

Compound		EC_{50}	Aspartic acid[a]		Mannose[a]		Ratio	
X	R	(μM)	Acc[b]	Inc[c]	Acc[b]	Inc[c]	Inc[d]	%[e]
Control		0	41	4.5	0.72	47	0.187	0
4-C_6H_5	H	>40	41	4.2	0.83	39	0.189	−1
2,3-Cl_2	CH_3	>40	42	4.1	0.90	35	0.185	1
4-$N(CH_3)_2$	H	>40	42	4.3	0.77	42	0.180	5
3-$N(CH_3)_2$	H	>40	42	4.2	0.84	35	0.168	13
3-OC_6H_5	H	27	41	4.2	0.77	37	0.166	15
H	H	27	41	4.4	0.82	35	0.162	17
2,4-Cl_2	H	14	40	4.8	0.71	40	0.149	26
2,3-Cl_2[f]	H	0.7	38	4.3	0.70	10	0.044	100
2-$O(CF_2)O$-3	H	0.2	37	4.2	0.69	8	0.038	104

[a] Mean results of four experiments. Variation in repetitions less than 5%.
[b] Accumulation in mycelium as percentage of label added.
[c] Incorporation into macromolecules as percentage of accumulation.
[d] Ratio between mannose and aspartic acid incorporated into macromolecules.
[e] Relative to fenpiclonil.
[f] Fenpiclonil.

Hence, under the growth conditions used, fenpiclonil (0.4 μM) inhibited incorporation of glucose into almost all macromolecular fractions (Table 4). The highest reduction was observed in the structural hyphal wall β-1,3-glucans of fractions I and II.

Glucan Synthetase Activity

β-1,3-Glucan synthesis is mediated by β-1,3-glucan synthetases in the plasma membrane (18, 22). Inhibition of activity of these enzymes would result in accumulation of its substrate UDPGlc (23). The concentration of UDPGlc was estimated by incubating mycelial suspensions with [2-^{14}C]uracil. After extraction the metabolites were quantified (Table 5). Upon fenpiclonil treatment, the internal uracil concentration considerably increased. The major part of the accumulated uracil was metabolized to UDPGlc and UDPGlcNAc. Upon incubation with fenpiclonil, a reduction of incorporation into UDPGlcNAc was noted.

Cell-free glucan synthetase activity of mycelial extracts was 4.3 nmol min^{-1} mg^{-1} protein. Activity could not be inhibited by fenpiclonil at concentrations ranging from 0.4 to 40 μM.

DISCUSSION

In liquid media with growth-limiting glucose concentrations, fenpiclonil (0.4 μM)

TABLE 3

Effect of Fenpiclonil (0.4 μM) on the Fate of the Radiolabel in [U-^{14}C]Mannose in Fusarium sulphureum *Isolate 1743 during 15 min of Incubation in a Medium Containing 100 μM Cold Glucose*[a]

Fraction	Control	Fenpiclonil[b]	
	%	%[c]	%[d]
Carbondioxide	65.2	62.0	95
$CHCl_3$/MeOH extract	24.0	32.7	136
PAW extract	2.8	1.8	64
Protein	2.0	1.4	70
Oligosaccharide	0.8	0.4	50
Residue	8.0	3.5	44
Accumulation	13.4[e]	10.5[e]	78

[a] Mean results of three experiments. Variation in repetitions less than 5%.
[b] Fenpiclonil added 15 min prior to addition of [U-^{14}C]mannose.
[c] Incorporation as percentage of label accumulated.
[d] Effect of fenpiclonil as percentage of control.
[e] Percentage of total label added.

inhibited the incorporation of glucose and mannose into various macromolecules. Incorporation of mannose was inhibited more strongly than that of glucose (Table 1). Under similar conditions, accumulation of these sugars and of various other substrates tested was only slightly influenced. This is in contrast to results previously reported for a growth-supporting glucose concentration, in which high concentrations of fenpiclonil (4 μ*M*) inhibited accumulation of a wide variety of substrates (2). Also in the present experiments relatively high concentrations of fenpiclonil aspecifically inhibited accumulation and incorporation of all substrates (Figs. 2A and 2B). Specific effects on sugar metabolism were corrected for aspecific effects by plotting the ratio between accumulated mannose and aspartic acid and between incorporated mannose and aspartic acid. These figures confirm that the accumulation ratio is barely affected, while the incorporation ratio is significantly reduced (Figs. 2C and 2D). This was not the case with the less-sensitive isolate 1743.1. Inhibition of mannose incorporation by various fenpiclonil analogues ranked with their fungitoxicity (Table 2). Therefore, the mode of action of fenpiclonil may be based on incorporation of sugars into macromolecules rather than on inhibition of accumulation of sugars and amino acids into mycelium as suggested previously (2).

The main biosynthetic pathways for various fungal macromolecules starting from glucose are shown in Fig. 3. One arrow does not necessarily indicate a single enzymatic step and the steps may be reversible. The major part of mannose in mycelium is present in saccharide side chains of glycoproteins (20) and can be marked by feeding mycelium with radiolabeled mannose (14). Incorporation of mannose into glycoprotein sugars was considerably inhibited by fenpiclonil (Table 3). However, the major part of the accumulated mannose 6-phosphate apparently isomerized to fructose 6-phos-

TABLE 4

Effect of Fenpiclonil (0.4 μM) on the Fate of the Radiolabel in [U-^{14}C]Glucose (100 μM) in Fusarium sulphureum *Isolate 1743 during 15 min of Incubation*[a]

Fraction	Control %[c]	Fenpiclonil[b] %[c]	Fenpiclonil[b] %[d]
CO_2	8.8	4.8	54
$CHCl_3$/MeOH Extract	60.9	77.4	127
SLS Extract	14.2	8.1	57
Fraction I	4.7	2.6	56
Protein	2.1	1.5	73
Glycan	2.6	1.1	42
Fraction II	4.9	2.5	52
Protein	0.6	0.7	116
Glycan	4.3	1.8	43
Fraction III	1.1	1.2	109
Protein	0.3	0.6	211
Glycan	0.8	0.5	68
Chitin	0.9	1.1	123
Accumulation	84.0[a]	71.6[a]	85
Recovery	98.7[e]	99.3[e]	

[a] Mean results of two experiments. Variation in repetitions less than 5%.

[b] Fenpiclonil added 15 min prior to addition of [U-^{14}C]glucose.

[c] Incorporation as percentage of radioactivity accumulated.

[d] Effect of fenpiclonil as percentage of control.

[e] Percentage of total label added.

TABLE 5

Effect of Fenpiclonil (0.4 μM) on the Fate of [2-^{14}C]Uracil (10 μM) in Fusarium sulphureum *Isolate 1743 during 15 min of Incubation*[a]

Metabolite	Control %[c]	Fenpiclonil[b] %[c]	Fenpiclonil[b] %[d]
Uracil	1.0 (7.8)	2.2 (19.7)	223
Uridine	0.44 (3.5)	0.18 (1.6)	40
Nucleotides	1.7 (13.1)	1.7 (15.5)	104
RNA	0.77 (6.0)	0.52 (4.6)	67
UDPGlc	2.5 (19.4)	2.4 (20.9)	95
UDPGlcNAc	6.5 (50.3)	4.3 (37.8)	66
Accumulation	12.8	11.3	88

[a] Mean results of two experiments. Variation in replicate values less than 5%.

[b] Fenpiclonil added 15 min prior to addition of uracil.

[c] Percentage of uracil added (percentage of uracil accumulated).

[d] Effect of fenpiclonil (percentage of control).

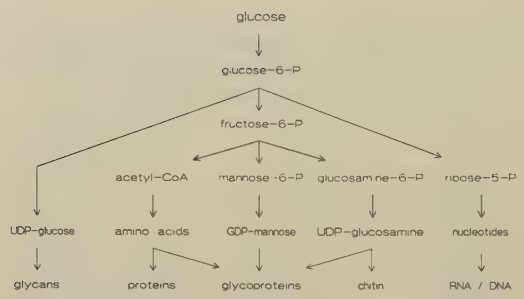

FIG. 3. *The main biosynthetic pathways of macromolecules in fungi* (*4, 5, 10, 11, 20, 24, 33, 36, 39*).

phate and glucose 6-phosphate (24), since a high proportion of accumulated mannose was metabolized to carbon dioxide, and a high proportion of macromolecules consisted of labeled glucose (Table 3). Fenpiclonil does not affect $^{14}CO_2$ release from [U-^{14}C]mannose, whereas the release from [U-^{14}C]glucose is inhibited by almost 50% (cf. Tables 3 and 4). This indicates that the site of inhibition is located in the metabolic pathway of glucose before the point of entry of mannose. Therefore, glucose was used in following experiments.

In these experiments, mycelial macromolecules were isolated and fractionated into water-soluble macromolecules (SLS extract) and cell wall macromolecules (fractions I–III and chitin) according to Sietsma and colleagues (11, 25). Cell walls of filamentous fungi primarily consist of glycoproteins and glycans embedded in a crystalline chitin microfibril matrix (26–29). The glycans generally have a β-1,3 and β-1,3-1,6 structure (10, 11). *F. sulphureum* does not contain β-1,4-glucans (21). The nature of compounds in the various fractions was partly verified and appeared comparable to data published for *Schizophyllum commune* (11). Fenpiclonil inhibited incorporation of glucose into almost all fractions obtained, but inhibition of incorporation into structural hyphal wall glycans (fractions I and II) was most significant (Table 4). Polymerization of UDPGlc is mediated by glucan synthetases located in the fungal plasma membrane (18, 22). However, activity of these enzymes is probably not directly affected by fenpiclonil, since cell-free polymerization could not be inhibited by the fungicide. Furthermore, *in vivo* inhibition of synthetase activity by fenpiclonil would result in accumulation of its substrate, UDPGlc (23). This was not the case (Table 5). In addition, depletion of structural glycans in the hyphal wall would result in morphological changes of the mycelium upon fenpiclonil treatment (30–32). This has not been observed (2).

Enzymes which mediate the polymerization of amino acids to proteins, nucleotides to DNA and RNA, and *N*-acetylglucosamine to chitin were not inhibited by fenpiclonil (2). Therefore, it is not likely that fenpiclonil interferes with the final polymerization steps of the various biosynthetic pathways; it is more likely that it interferes with early metabolization steps of glucose. A putative target site may be the phosphorylation of glucose, the first step in its metabolization. Glucose 6-phosphate is the precursor of various biosynthetic routes (Fig. 3) (33–35). Inhibition of glucose phosphorylation would lead to accumulation of nonphosphorylated sugars, which may explain the accumulation of radiolabel in the $CHCl_3/CH_3OH$ extract of fenpiclonil-treated mycelium (Tables 3 and 4). It would also explain the reduced levels of glycans (Table 4). A reduction in the glucose 6-phosphate content may also lower the ribose 5-phosphate content and explain the reduced glycosylation of uracil to uridine (Table 5). The normal biosynthetic route for pyridine nucleotides involves glycosylation of orotate to orotidylate before decarboxylation to uridine takes place (36, 37). A decrease in ribose 5-phosphate content may inhibit this route as well and would also result in reduced levels of uridine and UDP-activated sugar donors. Hence, inhibitory effects of fenpiclonil on initial steps of glucose metabolism may affect the biosynthesis of glycans in various ways (38, 39).

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Engineering Cyanobacterial Models Resistant to Bleaching Herbicides[1]

UTE WINDHÖVEL, BERND GEIGES, GERHARD SANDMANN, AND PETER BÖGER

Lehrstuhl für Physiologie and Biochemie der Pflanzen, Universität Konstanz, D-78434 Konstanz, Germany

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Enzymes catalyzing the dehydrogenation steps of the carotenoid biosynthetic pathway are target sites for bleaching herbicides. We introduced the gene *crtI*, coding for phytoene desaturase of the nonphotosynthetic bacterium *Erwinia uredovora* into the cyanobacterium *Synechococcus* PCC 7942, using it as a convenient experimental model for higher-plant transformation. The heterologous expression of the foreign gene in the transformants was demonstrated by Western blot analysis. The respective gene product CRTI, the enzyme phytoene desaturase, catalyzes the conversion of phytoene to lycopene via phytofluene, ζ-carotene, and neurosporene. It is highly resistant against various herbicides that affect either phytoene or ζ-carotene desaturase activity. Molar I_{50} values of carotenoid synthesis caused by various bleaching herbicides and determined at the intact cell revealed that the transformant strains exhibited strong cross-resistance toward the herbicides assayed. Resistance factors were more than 3 orders of magnitude greater than those of the control strain. © 1994 Academic Press, Inc.

INTRODUCTION

Carotenoids are yellow, red, and orange pigments present in all photosynthetic and many nonphotosynthetic organisms. In photosynthetic tissues they act as light-harvesting antennae and as protection against photooxidative destruction of the cells (1, 2). Inhibition of enzymes of carotenoid biosynthesis reduces the content of carotenoids which results in the destruction of chloroplast membranes and pigments (3).

Within the carotenoid biosynthesis pathway phytoene desaturase catalyzes the processing of the first C_{40} precursors and has been found to be inhibited by numerous chemically unrelated compounds (4). This has raised the interest of synthetic agrochemists, because of the opportunity to discover inhibitors of this enzyme which could be developed into commercial herbicides. Phytoene desaturase (PDS) was found to be highly homologous in oxygenic photosynthetic organisms. Except for a highly conserved region coding for the binding site of dinucleotides (FAD/NAD(P)), there is no homology to the phytoene desaturases from nongreen organisms (5, 6).

The phytoene desaturases from the cyanobacterium *Synechococcus* PCC 7942 and the nongreen phytopathogenic bacterium *Erwinia uredovora* also differ in their biological function. The *Erwinia* enzyme catalyzes four desaturation steps converting phytoene to lycopene via phytofluene, ζ-carotene, and neurosporene, while the *Synechococcus* enzyme catalyzes only two desaturation steps transforming phytoene to ζ-carotene. In *Synechococcus* the two desaturations leading from ζ-carotene to lycopene are catalyzed by an additional enzyme, ζ-carotene desaturase (7). By *in vitro* assays it could be demonstrated that the *E. uredovora* phytoene desaturase is not influenced by bleaching herbicides which strongly inhibit the phytoene or ζ-carotene desaturase from *Synechococcus* PCC 7942 (8).

In a preceding study we introduced the phytoene desaturase gene (*crtI* gene) from *E. uredovora* (9) into an "integration platform" located in the *metF* gene of the *Synechococcus* PCC 7942 genome (10) gener-

[1] This paper is dedicated to Prof. Dr. Achim Trebst on the occasion of his 65th birthday.

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ating transformants highly resistant to the bleaching herbicide norflurazon (11).

The present paper documents that these strains also exhibit strong cross-resistance against other chemically different herbicides that have been shown to inhibit phytoene or ζ-carotene desaturase (12).

Furthermore, we generated a new *Synechococcus* transformant in which the endogenous phytoene desaturase gene (*pds* gene) (13) is inactivated and substituted in part by the *Erwinia crtI* gene. This transformant is also very resistant to the bleaching herbicides.

MATERIALS AND METHODS

Organisms and growth conditions. The small-plasmid-cured *Synechococcus* PCC 7942 strain R2SPc and all transformant strains were grown in BG11 medium (14) buffered with 5 m*M* *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (Tes) (pH 8.0). For the selection on solid media, 1.5% (w/v) Bacto-Agar was added. The plates were maintained in an illuminated incubator (Rumed Rubarth Apparate GmbH, Hannover, Germany), which was gassed by an air-CO_2 mixture (90:10, v/v), at a temperature of 30°C, a light intensity of 50 μE m^{-2} s^{-1}, and 90% humidity. Liquid batch cultures were grown in culture vessels in a thermostat (Kniese-Edwards, Marburg) at 30°C. The cultures were illuminated with continuous fluorescent white light (60 μE m^{-2} sec^{-1}) and gassed with a mixture of CO_2 in air (98:2, v/v, 60 ml min^{-1}).

The transformant strain *Synechococcus* PCC 7942-PIM8 was grown and selected in the presence of 10 μg/ml streptomycin, strain -KOK in the presence of 10 μg/ml kanamycin, and strains -PIM8-BG0, -PIM8-BG1, and -PIM8-BG2 in the presence of 10 μg/ml kanamycin and 1 μg/ml ampicillin. Taking into account the methionine auxotrophy of strains *Synechococcus* PCC 7942-PIM8, -PIM8-BG0, -PIM8-BG1, and -PIM8-BG2, the media were supplemented with 30 μg/ml L-methionine.

Escherichia coli DH5α (Bethesda Research Laboratories, Gaithersburg, MD), which was used for plasmid propagation, was grown as described (15). For selection of plasmids, 50 μg/ml ampicillin or kanamycin was added to the growth media.

DNA manipulations. These were performed using standard techniques (15, 16). Transformation of *Synechococcus* PCC 7942 strains was carried out by a modification (11) of a previously described procedure (17).

For Southern hybridizations the Digoxygenin Nonradioactive DNA Labeling and Detection Kit from Boehringer (Mannheim, Germany) was used following the supplier's protocol.

Construction of plasmids. Plasmids p*pds*del35, pBG0, pBG1, and pBG2 used for transformation of *Synechococcus* PCC 7942-PIM8 or as a source for the construction of plasmid pKOK have been described earlier (7, 11).

For construction of pKOK, the region between the *Hin*dII site at base pair (bp) 465 and the *Hin*dIII site at bp 1565 of p*pds*del35 coding for the 3′ end of the *Synechococcus pds* gene (numbers of the nucleotides refer to the previously published sequence (13)) was replaced by the 3.1 kilobase (kb) *Hin*dII/*Hin*dIII fragment of pBG1 which carries the *crtI* gene of *Erwinia uredovora* downstream of the *npt*II gene from transposon Tn*5* (18) (Fig. 1).

Immunodetection of E. uredovora phytoene desaturase (CRTI). Protein extracts from *Synechococcus* PCC 7942 cells were obtained by a modified procedure (19). Twenty-milliliter aliquots from *Synechococcus* PCC 7942 liquid cultures were harvested at an optical density measured at 730 nm (OD_{730}) of about 3 by centrifugation at 4000*g* at room temperature. The pelleted cells were resuspended in 2 ml lysis buffer (2 mg/ml lysozyme in 10 m*M* Tes/NaOH, pH 7.0, 600 m*M* sucrose, and 2 m*M* EDTA). After incubation for 2 hr at 30°C, the cells were disrupted with a French press (140 MPa). Unbroken cells were removed by centrifugation.

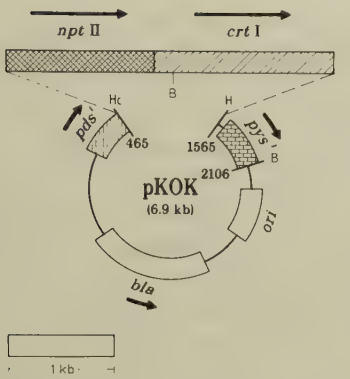

FIG. 1. *Schematic presentation of plasmid pKOK. The numbers indicating base pairs are taken from Chamovitz et al. (13). Arrows refer to transcriptional orientation. Cleavage sites for restriction enzymes are indicated as follows: B, BamH1; H, HindIII; Hc, HincII. Abbreviations used: bla, gene coding for resistance to ampicillin; crtI, gene coding for the phytoene desaturase of Erwinia uredovora; nptII, gene coding for resistance to kanamycin; pds, gene coding for the phytoene desaturase of Synechococcus PCC 7942; pys, gene coding for the phytoene synthase of Synechococcus PCC 7942; ori, origin of replication. A dash at the notations designates incomplete genes. The insert is harbored in ppdsdel35 (7).*

Proteins were electrophoretically separated by SDS–PAGE (20) and subsequently transferred to a nitrocellulose filter (21). The immunodetection of CRTI was performed using a polyclonal antibody raised against this enzyme (22) as previously described (11).

Carotenoid analyses. Liquid cultures of *Synechococcus* PCC 7942 strains were grown for 2 days until a density of 1 to 1.5 μl packed cell volume (pcv)/ml suspension was reached. Then, 20-ml aliquots were harvested by centrifugation. To determine total carotenoids, the pelleted cells were extracted (20 min, 65°C) with methanol containing 6% (w/v) KOH, partitioned against 10% diethyl ether (b.p. 35–80°C), and the absorption of the ether phase at 445 nm was measured. For calculation an overall extinction coefficient $E^{1\%}_{1cm}$ (i.e., optical density of 1 g carotenoid in 100 ml solution in a 1-cm light-path spectrophotometer cuvette) of 2500 was used (23).

To investigate the herbicide resistance of the *Synechococcus* PCC 7942 transformant strains, bleaching herbicides dissolved in methanol were added to the media. The molar concentrations of 50% inhibition (I_{50} value) was determined by a graphical procedure (24).

Chemicals. Norflurazon (SAN 9789; 4-chloro-5-methylamino-2-(3-trifluoromethylphenyl)-pyridazin-3(2*H*)one) was a gift from Sandoz AG (Basel, Switzerland); fluridone (EL-171; 1-methyl-3-phenyl-5-(3-trifluoromethylphenyl-4(1*H*)-pyridone) was from Lilly Research Labs, (Greenfield, IN); fluorochloridone (R-40244; 1-(*m*-trifluoromethylphenyl)-3-chloro-4-chloromethyl-2-pyrrolidinone) was from ICI/Stauffer (Richmond, CA); flurtamone (RE 40885; 5-(methylamino)-2-phenyl-4-(3-trifluoromethylphenyl)-3(2*H*)-furanone) was from Chevron Chemical Co., (Richmond, CA); J852 (4-(3-methylpropoxy)-2-isopropylamino-6-methylpyrimidine) was from ICI Agrochemicals, Bracknell, England; and LS 80707 (ethyl-*cis*-5-methyl-6-ethyl-2-phenyl-5,6-dihydropyran-4-one-3-carboxylate) was given to use from Rhône-Poulenc Agrochimie (Lyon, France).

RESULTS

Generation of transformant strains. We have recently described two transformant *Synechococcus* PCC 7942 strains carrying the phytoene desaturase gene of *Erwinia uredovora* (*crtI* gene) under the control of two different promoters in the so-called "integration platform" in the genome of *Synechococcus* PCC 7942-PIM8. This strain is a derivative of the small-plasmid-cured strain PCC 7942-R2SPc (10, 11). The platform consists of the streptomycin-resistance gene (*aad* gene) flanked by the promoterless ampicillin-resistance gene (*bla* gene) and the origin of replication of plasmid pBR322. It is integrated in the *metF* gene, which is involved in methionine biosynthesis. Provided that the growth media are supplemented with methionine, the "platform" does not affect the viability of the cyanobacteria. Transformation of this

strain with any pBR322-derived donor plasmid occurs by recombination between the *bla* and *ori* sequences of the "platform" and the donor plasmid. This leads to restoration of the *bla* gene and concurrent replacement of the *aad* gene by the insert of the donor plasmid (10).

In strain *Synechococcus* PCC 7942-PIM8-BG1, the *crtI* gene and the *npt*II gene from transposon Tn*5*, conferring resistance to kanamycin, are cotranscribed from the promoter of the *npt*II gene residing on the left insertion element IS*50*L of transposon Tn*5*. In strain PIM8-BG2, expression of the *crtI* gene occurs under the control of the promoter of the *psbA* gene from the microalga *Bumilleriopsis filiformis* (25) (Fig. 2). Thus, both transformant strains carry the *crtI* gene in addition to the endogenous

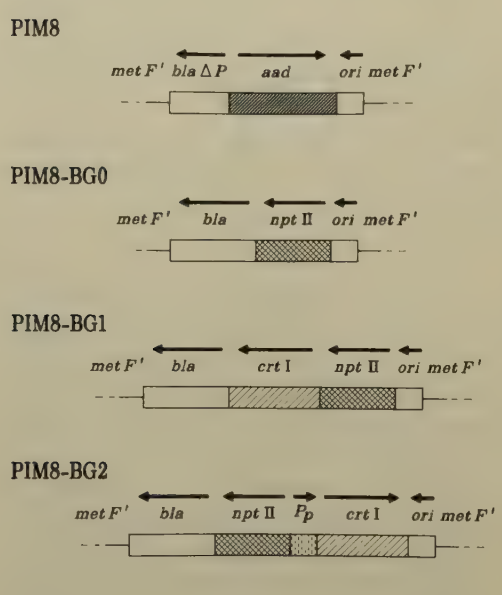

FIG. 2. *Genomic region containing the "integration platform" in various Synechococcus transformants. Abbreviations as used in Fig. 1 with the addition of: aad, gene coding for streptomycin resistance; blaΔP, promoterless bla gene; metF, gene involved in methionine biosynthesis of Synechococcus PCC 7942; P_p, promoter of the psbA gene of Bumilleriopsis filiformis. The correct integration of the inserts has recently been demonstrated by Southern blot analysis (11).*

pds gene in their genome. These genes code for two different types of phytoene desaturases. Strain *Synechococcus* PCC 7942-PIM8-BG0, harboring only the *npt*II gene in the "integration platform" was used as the control strain (Fig. 2).

We have constructed a new plasmid, pKOK, by cloning the insertion fragment of pBG1, carrying the *crtI* gene colinear to the *npt*II gene, into plasmid p*pds*del35 between the 5′ end of the *pds* gene and the 5′ end of the phytoene synthase gene (*pys* gene) of *Synechococcus* PCC 7942. Genes *pds* and *pys* are located adjacent to each other in the cyanobacterial genome. Thus, after transformation of *Synechococcus* PCC 7942-R2SPc with pKOK a "double crossover event" is expected to take place between the *Synechococcus* genome and this plasmid by recombination of the homologous sequences of both the 5′ region of the *pds* gene and the 5′ region of the *pys* gene. Therefore, in the genome of the resulting transformant strain *Synechococcus* PCC 7942-KOK, the *pds* gene is destroyed and replaced by the insertion fragment (Fig. 3A).

Correct integration of the insertion fragment was checked by Southern hybridization experiments. The molecular mass of the hybridizing fragments match with the calculated ones, indicating correct integration of the insert of pKOK into the *Synechococcus* genome (Fig. 3B).

Synechococcus PCC 7942 may harbor up to 10 identical chromosomes in its genome (26). To demonstrate the absence of any copy of the *pds* gene in the genome of *Synechococcus* PCC 7942-KOK, Southern hybridizations were performed with an *Nco*I fragment as a probe comprising the region between bp 618 and 1218 of the *pds* gene. In experiments with this hybridization probe, a clear hybridization signal was obtained with *Synechococcus* PCC 7942 R2SPc genomic DNA, while no detectable signal was obtained even with 20-fold concentrations genomic DNA from *Synechococcus* PCC 7942-KOK (data not shown). Moreover,

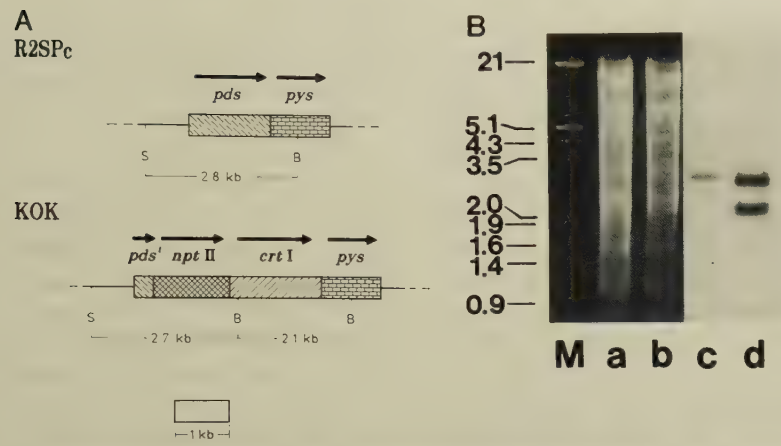

FIG. 3. *Region containing the pds and pys genes in Synechococcus PCC 7942-R2SPc and PCC 7942-KOK; and Southern blot analysis. (A) The region containing pds and pys gene and the sizes of fragments that hybridize with pKOK in the Southern blot analysis of Fig. 3B are given. Cleavage sites of the restriction enzymes were known from recent publications (9, 13). Abbreviations as in Fig. 1, and "S" indicates the cleavage site of restriction enzyme SalI. (B) Southern blot analysis: A 0.7% agarose gel was run with the following samples: BamHI/SalI double-digest of total genomic DNA from Synechococcus PCC 7942-R2SPc (a) and Synechococcus PCC 7942-KOK (b). HindIII/EcoRI double-digested phage λ DNA was used as molecular mass marker (M). The DNAs were blotted to a nitrocellulose filter and hybridized with digoxygenin-labeled pKOK as probe. The hybridization signal obtained with DNA from Synechococcus PCC 7942 R2SPc is shown in lane c and the one obtained with DNA from Synechococcus PCC 7942-KOK is in lane d.*

these results show that plasmid pKOK has not been integrated into the cyanobacterial genome by a "single crossover event" because in that case, in the above-described Southern hybridization experiments, a hybridization signal should be obtained.

Immunodetection of heterologously expressed CRTI. Western blot analysis was performed using a polyclonal antibody raised against *E. uredovora* phytoene desaturase (22). The heterologously expressed CRTI protein could be detected in cell extracts of transformant strains *Synechococcus* PCC 7942-PIM8-BG1, -PIM8-BG2, and strain -KOK (Fig. 4). As can be estimated from the intensity of the immunoreactive bands strains *Synechococcus* PCC7942-PIM8-BG1 and PCC 7942-KOK express comparatively larger amounts of CRTI than PCC 7942-PIM8-BG2.

The amounts of CRTI expressed by *Synechococcus* PCC 7942-PIM8-BG1 and -KOK seem to be in the same range. As expected, in cell extracts of controls *Synechococcus* PCC 7942-PIM8, PCC 7942-PIM8-BG0, and PCC 7942-R2SPc, no immunoreaction was detected.

Analysis of resistance of transformant strains against bleaching herbicides. Syn-

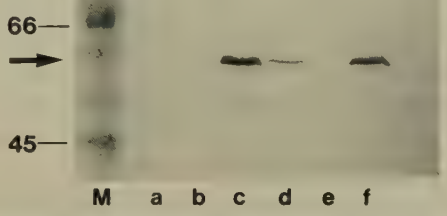

FIG. 4. *Immunodetection of the Erwinia CRTI enzyme. Western blot analysis was performed with cell extracts of various Synechococcus PCC 7942 strains with an antiserum raised against the phytoene desaturase of Erwinia uredovora. The arrow points to the heterologously expressed CRTI with an apparent molecular weight of about 54 kDa. M, molecular weight marker; a, Synechococcus PCC 7942 PIM8; b, Synechococcus PCC 7942-PIM8-BG0; c, Synechococcus PCC 7942-PIM8-BG1, d, Synechococcus PCC 7942-PIM8-BG2; e, Synechococcus PCC 7942-R2SPc; f, Synechococcus PCC 7942-KOK. Numbers refer to apparent molecular weight.*

echococcus strains were grown in the presence of various concentrations of chemically nonrelated bleaching herbicides, which affect the desaturation of either phytoene or ζ-carotene. The herbicides norflurazon, fluridone, fluorochloridone, and flurtamone inhibit phytoene desaturation, whereas J852 and LS80707 are ζ-carotene desaturase inhibitors. After 2 days of cultivation in the light, the I_{50} values for inhibition of formation of colored carotenoids were determined.

In a preceding study, we reported that strains *Synechococcus* PCC 7942-PIM8-BG1 and PCC 7942-PIM8-BG2 are resistant to norflurazon. As can be seen from Table 1 strain *Synechococcus* PCC 7942-KOK is also strongly resistant against norflurazon. Moreover, compared to the control strain PCC 7942-PIM8-BG0, the three strains containing the foreign *crtI* gene exhibit strong cross-resistance against the above-mentioned phytoene desaturase as well as against ζ-carotene desaturase inhibitors. The degree of resistance of *Synechococcus* PCC 7942-PIM8-BG2 against each of the herbicides is clearly lower than those of PCC 7942-PIM8-BG1 and PCC 7942-KOK. Due to limited solubility of the herbicides, I_{50} values of the latter two strains against norflurazon, fluridone, flurtamone, and J852 could not be precisely measured. With herbicides present at 10^{-4} *M* concentrations, little or no bleaching of the cells occurred. The I_{50} value of *Synechococcus* PCC 7942-KOK was slightly higher against fluorochloridone and about 10-fold higher against LS 80707 than that of *Synechococcus* PCC 7942-PIM8-BG1.

DISCUSSION

At present, two main approaches are followed to genetically engineer resistance against herbicides. On the one hand, genes coding for detoxifying enzymes are introduced into plants, whereas, on the other hand, transformation is performed with genes coding for target enzymes that are resistant to the corresponding herbicide (27).

In a former study diverse norflurazon-resistant *Synechococcus* PCC 7942 strains were isolated (28). Sequence analyses of the *pds* gene in the mutants revealed that they carried different single point mutations in the coding region and in one case a deletion in its putative promoter (13, 29). While the former led to single amino acid substitutions, the latter obviously resulted in a stronger promoter, which was manifested by increased production of PDS. Besides resistance to norflurazon, for which they had been selected, some of the mutants were found to exhibit cross-resistance to the PDS inhibitors, fluridone and fluorochloridone (29).

Based on *in vitro* experiments it was already known that the *Erwinia uredovora* phytoene desaturase is not influenced by herbicides that inhibit either the phytoene

TABLE 1

I_{50} Values [Molar] for in Vivo Inhibition of Carotenogenesis in Synechococcus Transformants by Bleaching Herbicides Affecting (a) Phytoene Desaturase or (b) ζ-Carotene Desaturase

Strain	Norflurazon[a]	Fluridone[a]	Fluorochloridone[a]	Flurtamone[a]	J852[b]	LS 80707[b]
PIM8-BG0	9.4×10^{-8}	3.4×10^{-8}	1.1×10^{-7}	3.2×10^{-8}	4.9×10^{-8}	7.1×10^{-7}
PIM8-BG2	2.3×10^{-6}	7.8×10^{-5}	3.6×10^{-5}	4.8×10^{-5}	3.5×10^{-5}	3.3×10^{-6}
	(24)	(2300)	(330)	(1500)	(710)	(5)
PIM8-BG1	$>10^{-4}$	$>10^{-4}$	8.1×10^{-5}	$>10^{-4}$	$>10^{-4}$	3.6×10^{-5}
	($>10^3$)	($>10^3$)	(740)	($>10^3$)	($>10^3$)	(51)
KOK	$>10^{-4}$	$>10^{-4}$	9.8×10^{-5}	$>10^{-4}$	$>10^{-4}$	3.4×10^{-4}
	($>10^3$)	($>10^3$)	(890)	($>10^3$)	($>10^3$)	(480)

Note. Data in parentheses indicate the resistance factor of the transformant strains toward the bleaching herbicides with respect to the control strain *Synechococcus* PCC 7942-PIM8-BG0.

desaturase or the ζ-carotene desaturase of green organisms (8). Therefore, we introduced three different constructs, containing the *crtI* gene of *E. uredovora* into the cyanobacterium *Synechococcus* PCC 7942 to investigate whether heterologous expression of the corresponding enzyme leads to resistance to these bleaching herbicides in an oxygenic photosynthetic host organism. Our data indicate that the *Erwinia* phytoene desaturase provides a much better means to engineer herbicide resistance than the mutated endogenous *Synechococcus* enzyme. The I_{50} value of strain *Synechococcus* PCC 7942-NFZ49 was 7.7×10^{-6} *M* exhibiting the highest degree of resistance against this herbicide (28) which is 2 orders of magnitude less than those obtained by strains PCC7942-PIM8-BG1 and -KOK. Moreover, the transformant strains described in our study exhibited strong cross-resistance not only against bleaching herbicides inhibiting phytoene desaturase but also toward ζ-carotene desaturase inhibitors. A further advantage of using the naturally resistant *Erwinia* phytoene desaturase instead of a mutated endogenous *pds* gene is that expression of the mutated gene led to a reduced efficiency of the phytoene desaturation reaction (29), which was not the case after introduction and heterologous expression of *crtI* (11).

Since cyanobacteria carry out a plant-type oxygenic photosynthesis, these findings can serve as a model to generate herbicide-resistant crop plants. The first experiments in which the *crtI* gene was introduced into tobacco, have been described recently (30). The transformed tobacco had a significant level of norflurazon resistance.

The immunoblot (Fig. 4) reveals that *Synechococcus* PCC 7942-PIM8-BG2 expresses a lower amount of CRTI than *Synechococcus* PCC 7942-PIM8-BG1 and -KOK. *Synechococcus* PCC 7942-PIM8-BG2 is also less resistant to bleaching herbicides than the latter two strains, indicating that the degree of resistance depends on the amount of foreign enzyme expressed.

Due to the high level of resistance for phyloene desaturase, precise I_{50} values of *Synechococcus* PCC 7942-PIM8-BG1 and -KOK could be determined only for the phytoene desaturase inhibitor fluorochloridone and the ζ-carotene desaturase inhibitor LS80707. Although both strains seem to express almost equal amounts of CRTI, the I_{50} value of *Synechococcus* PCC 7942-KOK is slightly higher for fluorochloridone and about 10-fold higher for LS 80707 than that of -PIM8-BG1. Thus, in the strain -KOK the foreign enzyme obviously is more active than in strain -PIM8-BG1. This is probably due to the fact that in strain -KOK, in contrast to strain -PIM8-BG1, the endogenous phytoene desaturase gene is deleted. The CRTI enzyme alone is instrumental for carotene biosynthesis. Presumably, this strain integrates a relatively higher amount of this enzyme into its photosynthetic membranes than -PIM8-BG1.

For the phytoene desaturase from *Rhodobacter capsulatus,* in addition to the enzymatic activity a structural function in the formation of the photosynthetic antennae is discussed (31). If the *Synechococcus* enzyme plays a comparable role, then the substitution of the *pds* gene in the genome of *Synechococcus* PCC 7942-KOK by the *crtI* gene indicates that the *Erwinia* enzyme can take over the biochemical as well as the structural function of the endogenous phytoene desaturase.

The phytoene desaturase of *E. uredovora* catalyzes four desaturation steps converting phytoene to lycopene. In photosynthetic oxygenic cells these steps are catalyzed by two enzymes, phytoene desaturase and ζ-carotene desaturase. Accordingly, it would be interesting to investigate whether the gene coding for the ζ-carotene desaturase can also be replaced by the bacterial *crtI* gene possibly leading to a further increase of resistance against bleaching herbicides. At the moment this gene (*zds* gene) has been isolated only from *Ana-*

baena PCC 7120 (32), but not from *Synechococcus*. As could be demonstrated by Southern hybridization experiments, the *Anabaena zds* gene exhibits no detectable homology with genomic DNA from *Synechococcus* PCC 7942 (33). Therefore, experiments to inactivate the endogenous ζ-carotene desaturase gene of *Synechococcus* could not be performed as yet.

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Basis of Selectivity of Triflusulfuron Methyl in Sugar Beets (*Beta vulgaris*)

V. A. WITTENBACH, M. K. KOEPPE, F. T. LICHTNER, W. T. ZIMMERMAN, AND R. W. REISER

DuPont Agricultural Products, Stine-Haskell Research Center, Newark, Delaware 19714

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Triflusulfuron methyl (methyl 2-[[[[[4-(dimethylamino)-6-(2,2,2-trifluoroethoxy)-1,3,5-triazin-2-yl]amino]carbonyl]amino]sulfonyl]-3-methylbenzoate), formerly DPX-66037, is a new selective postemergence sulfonylurea herbicide for the control of annual and perennial broadleaf weeds and grasses in sugar beets (*Beta vulgaris*). Similar to other sulfonylureas, the site of action of triflusulfuron methyl is acetolactate synthase (ALS), an enzyme in branched-chain amino acid biosynthesis. This herbicide is unique in this class in its safety on sugar beets, a crop that has often been used as an indicator of residual sulfonylureas in soil. We investigated the mechanism of selectivity by comparing the response of sugar beets with that of sensitive weeds (*Brassica napus, Matricaria inodora,* and *Veronica persica*) and a moderately tolerant weed (*Chenopodium album*). Studies of foliar uptake and *in vitro* inhibition of ALS across these species discount differential uptake or active site sensitivity as the basis for sugar beet tolerance to this herbicide. However, a good correlation between metabolism and plant tolerance does exist. Sugar beets metabolize triflusulfuron methyl very rapidly (half-life of <1 hr) while *C. album* has an intermediate rate (half-life of 7 hr) and the sensitive weeds have slow rates of metabolism (half-lives of >35 hr). The initial metabolism of triflusulfuron methyl in sugar beets involves nucleophilic attack by glutathione at the urea carbonyl group producing the *S*-carbamoyl glutathione conjugate plus 7-methylsaccharin and its free acid. The 2,6-disubstituted sulfonamide appears essential for this metabolism, but surprisingly even minor changes on the triazine ring have a profound effect on sugar beet tolerance. Although the data strongly suggest enzymatic involvement, attempts to determine if glutathione-*S*-transferase was involved proved inconclusive. © 1994 Academic Press, Inc.

INTRODUCTION

Triflusulfuron methyl (methyl 2-[[[[[4-(dimethylamino)-6-(2,2,2-trifluoroethoxy)-1,3,5-triazin-2-yl]amino]carbonyl]amino]sulfonyl]-3-methylbenzoate), formerly DPX-66037, is a sulfonylurea herbicide for weed control in sugar beets (*Beta vulgaris*). It is a highly active postemergence herbicide that controls a diverse spectrum of broadleaf weeds and also has activity on a range of grasses (1). In addition to its low use rate of 10 to 20 g a.i./ha, it is a short-residual herbicide, which breaks down rapidly in soil via chemical and to a lesser degree microbial mechanisms.

Similar to other sulfonylurea herbicides, the mechanism of action of triflusulfuron methyl is inhibition of acetolactate synthase (2, 3), an enzyme in branched-chain amino acid biosynthesis. This herbicide is unique among commercial sulfonylureas in its safety on sugar beets, a crop that has often been used as an indicator of residual sulfonylureas in soils. Because of the unique tolerance of sugar beets to triflusulfuron methyl, we have studied the mechanism of selectivity. In this paper we report the results of studies on plant and enzyme sensitivity, foliar uptake, and metabolism of [^{14}C]triflusulfuron methyl.

MATERIALS AND METHODS

Chemicals. [*Triazine*-^{14}C]- and [*ester carbonyl*-^{14}C]triflusulfuron methyl (1.48 and 1.51 GBq/mmol, respectively) were synthesized by New England Nuclear. The radiochemical purity of the compounds used in these experiments was >97% as determined by HPLC. Chromatographic standards of triflusulfuron methyl, the triazine

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amine, and *N*-desmethyl triazine amine were synthesized by DuPont Agricultural Products using published procedures (4–6). Oxidized glutathione (GSSG) was obtained from Aldrich Chemical Co.

Instrumentation. Nuclear Magnetic Resonance (NMR) spectra were obtained on a Bruker AM300 (300 MHz) spectrometer. Fast atom bombardment (FAB) mass spectra and LC/MS analyses were carried out on a Finnigan MAT 900 mass spectrometer system. For LC/MS, samples were dissolved in 50/50 acetonitrile/water and 0.1 μl was injected into a packed capillary LC column with a Valco C14W injection valve. An LC Packings C18 column (15 cm × 0.32 mm i.d.) was used with an acetonitrile/water (pH 3, formic acid) mobile phase at flow rates of 1–2 ml/min. Glycerol (5%) was added to the mobile phase to serve as a liquid matrix. An Isco CV4 uv detector was used on-line between the column and the mass spectrometer at 238 nm. The ion source temperature was 55°C. The mass range scanned was generally 100–1000.

Plant growth and treatment. Sensitivity to the herbicide was evaluated on sugar beet (*B. vulgaris*), *Chenopodium album, Matricaria inodora, Brassica napus,* and *Veronica persica* grown under greenhouse conditions. Plants were grown in Sassafras sandy loam soil (pH 6.7, 0.8% O.M.) in 10-cm fiber pots in a greenhouse maintained at 16–22°C. Plants at the 2–4 leaf stage were sprayed with a wide range of rates (4–500 g/ha) of triflusulfuron methyl formulated in a solution consisting of acetone/water/glycerin/Tween 20 (91.4/4.2/4.2/0.2, vol). Treated plants were returned to the greenhouse, watered as needed, and herbicidal injury was evaluated 16 days after treatment. Injury was visually estimated as percentage growth reduction relative to untreated plants. The data were subjected to linear regression analysis, and inverse prediction was performed to calculate the mean application rates (g/ha) causing 50% growth reduction (GR_{50} values) relative to untreated controls.

Acetolactate synthase extraction and assay. Acetolactate synthase (ALS, EC 4.1.3.18) was extracted from seedlings of each species at the same stage of growth that the plants were used for the uptake and metabolism studies. The enzyme extraction and assay protocols were the same as those described by Ray (3).

Uptake and metabolism studies in leaves. Plants used in these studies were grown under the same conditions as those used to evaluate herbicidal activity. The stage of growth at the time of treatment was three to four leaves for sugar beet, four leaves for *B. napus,* four leaves for *V. persica,* six to eight leaves for *M. inodora,* and five to six leaves for *C. album.* The uptake and metabolism studies were carried out in a growth chamber set at 18°C with a 14-hr day at a light density of 300 $\mu E/m^2/sec$. Plants were transferred from the greenhouse to the chamber 2 to 3 days prior to treatment. Herbicide uptake studies were initiated by foliar applications of [*triazine*-^{14}C]triflusulfuron methyl using a microsprayer designed for uniform droplet application with small quantities of compounds (7). The compound was formulated in water plus 0.1% Citowett surfactant and applied as a broadcast spray at 10 g/ha in a volume equivalent to 150 liter/ha (150 to 800 Bq/plant, depending on species). The sprayer and plants were completely enclosed in a glove box during application. The nozzle was set to deliver droplets with an average diameter of 300 μm.

Following application, the plants were transferred to the growth chamber and harvested 5 and 20 hr after treatment by excising the shoots at the soil level. The shoots were thoroughly washed by dipping and rinsing with 50% acetone/50% water. Total radioactivity in the rinsate was determined by liquid scintillation counting (LSC). The washed plants were dried and combusted to determine the quantity of ^{14}C in the tissue. Percentage uptake was calculated as the tissue radioactivity divided by the sum of the tissue and rinsate radioactivity.

Shoot explants were used for the metabolism study, and [*triazine*-^{14}C]triflusulfuron methyl was applied as a pulse through the transpiration stream. This system allowed a controlled comparison of metabolism rates among plant species without the complications of cuticular uptake, adjuvant effects on wetting, leaf surface area, and spray uniformity which are introduced by foliar applications. At the time of treatment, shoots were excised under water and the explants were then placed in quarter-strength Hoagland's solution (8) containing 10 ppm of [*triazine*-^{14}C]triflusulfuron methyl (30 kBq/ml). Explants were allowed to take up the compound until uptake reached ~1 μg/g fresh wt (1–2 hr), so that the initial concentration of herbicide within each species was approximately equal. At the end of the uptake period, the explants were transferred to solutions containing only quarter-strength Hoagland's. Plants were then harvested at various times up to 48 hr and placed in a −80°C freezer until extraction (Method 1) and analysis (HPLC Method 1). Results are reported as the means of two experiments (each time point represents three to five plants per experiment). Similar experiments were repeated with the [*ester carbonyl*-^{14}C]triflusulfuron methyl in sugar beets (extraction Method 1, HPLC Method 2).

Metabolism studies in roots. Sugar beet plants used in these studies were grown in sand to have clean root material. Plants in the three- to four-leaf stage were removed from the sand, and the roots were washed free of sand. The foliage was removed and the roots were placed in petri dishes containing 35 ml of quarter-strength Hoagland's nutrient solution plus [*triazine*-^{14}C]- or [*ester carbonyl*-^{14}C]triflusulfuron methyl (330 and 400 kBq, respectively). The stems were held above the solution using stainless steel screens. The roots were allowed to take up the solution for 1 hr. After the uptake period, unabsorbed [^{14}C]triflusulfuron methyl was washed from the roots by gently shaking twice in 35 ml of chilled quarter-strength Hoagland's nutrient solution (5–10 min each time). The roots were then placed in petri dishes with moist filter paper and incubated in the dark at room temperature. Roots were harvested at 0, 1, 2, and 6 hr, blotted dry, and immediately frozen in a −80°C freezer until extraction (Method 2) and analysis (HPLC Method 1 or 2 for [*triazine*-^{14}C]- or [*ester carbonyl*-^{14}C]triflusulfuron methyl, respectively). This experiment was run in duplicate.

Effect of a glutathione-S-transferase inhibitor on metabolism. Formulated tridiphane was applied as a foliar spray to sugar beets at 1000 g a.i./ha, and 24 hr later treated and untreated leaves were pulsed with [*triazine*-^{14}C]triflusulfuron methyl for 1.5 hr and chased for 0, 1, 2, 6, 10, and 26 hr as reported by Zorner *et al.* (9). Samples were extracted (Method 2) and analyzed by HPLC (Method 2) to determine the effect of the inhibitor of glutathione-*S*-transferase (GST) on metabolism.

Plant extraction and analysis. Two methods were used in the above experiments:

Method 1: Shoot explants (1–4 g fresh wt) were placed in 50-ml centrifuge tubes and extracted with 15 ml of 50% acetone/50% 0.1 *M* ammonium carbonate (v/v) using a Polytron homogenizer (Brinkmann Instruments). Extracts were centrifuged for 25 min at 2000*g* and the supernatant fluids were removed and saved. The pellets were extracted two more times with extraction solvent and the supernatants from the three extracts were combined. The combined supernatant fluids were concentrated under vacuum at 45°C on a rotary evaporator until the volume was reduced to approximately 5 ml. Extraction efficiencies were >90%. The final volume of each sample was adjusted to 10 ml with water and 1 ml acetonitrile and was analyzed by HPLC.

Method 2: Each sugar beet leaf sample was placed in a 2-ml vial and kept frozen under liquid nitrogen. The tissue was chopped using a small spatula and 0.5 ml of chilled glass beads (1 mm diameter) was added to the vial. The vial was shaken on a

bead mill (BioSpec Products, Mini-Bead Beater) for 60 sec to further break up the tissue. One milliliter of acetone/water (80/20, v/v) was added and the vial was shaken twice for 90 sec each time to extract the ^{14}C residues. The vial was microfuged at 14,000 rpm for 5 min to pellet the beads and plant pulp. The clear green extract was removed and analyzed by LSC and HPLC. Extraction efficiencies were >90%.

Chromatography conditions. HPLC separations were performed on either a Hewlett–Packard HP1090 liquid chromatograph equipped with a diode array detector in-line with a Raytest Ramona-5-LS radiodetector with a 0.5 ml CaFl cell or a Perkin–Elmer Series 4 chromatograph equipped with a variable wavelength detector in-line with a Radiomatic CT Flow One radiodetector with a 2.5 ml liquid cell. Ultraviolet chromatograms were obtained for nonradiolabeled metabolite standards at 254 nm and cochromatographed with radiolabeled metabolites from plant extracts using the radiodetector. The binary mobile phases were A = H_2O + 0.03% phosphoric acid or 0.2% formic acid and B = acetonitrile + 0.03% phosphoric acid or 0.2% formic acid. Each separation method described below was operated at a flow rate of 1.5 ml/min and a column temperature of 40°C using a DuPont Zorbax Rx-C8 reverse-phase column (4.6 × 250 mm). The separation methods are defined below.

Method 1: Linear Gradient

Time	% A	% B
0	80	20
20	40	60
22	40	60
23	80	20

Method 2: Linear Gradient

Time	% A	% B
0	100	0
1	100	0
31	40	60
36	40	60
37	100	0

Purification of 6-min metabolite. A major metabolite, eluting at 6 min by HPLC (Method 1), was purified from sugar beet leaves for identification by mass spectrometry. Thirty sugar beet seedlings were excised at the base of the petiole of the first two true leaves and placed in quarter-strength Hoagland's nutrient solution containing 75 ppm [*triazine*-^{14}C]triflusulfuron methyl (148 MBq/mmol; diluted to this lower specific activity with nonradiolabeled triflusulfuron methyl). The excised leaves were held in the lab at room temperature and under fluorescent lights and allowed to take up this solution for 2 hr. The leaves were then transferred to untreated Hoagland's nutrient solution for an additional 2 hr to allow metabolism of [^{14}C]triflusulfuron methyl.

The treated leaves (12.0 g) were frozen and macerated in an Omni mixer within a stainless steel cup for 1 min. Then, 25 ml of 80% acetone/20% water (v/v) was added to the cup and the leaves were extracted for 2 min. The plant residue was removed from the extract by centrifugation and reextracted as above. The combined extracts were rotary evaporated at 35°C to remove acetone, leaving approximately 10 ml of dark green aqueous extract. This aqueous extract was adjusted to pH 3 using acetic acid to precipitate plant pigments. The precipitate was pelleted by centrifugation and the clear pale yellow supernatant extract was neutralized to pH 6.0 using 2.5 *M* KOH. The extract was concentrated to 1.5 ml by rotary evaporation and centrifuged again due to precipitation during concentration.

The 6-min metabolite of triflusulfuron methyl was purified by HPLC using an Rx-C8 column and elution Method 1 (see above). Six 250-μl sample aliquots were chromatographed and fractions were collected at 0.5-min intervals (0.75 ml/injection) into the same set of vials. Under our conditions, the 6-min metabolite eluted quantitatively in fractions 12 and 13 (see Fig. 3 for a representative chromatogram).

These fractions were combined and concentrated to about 2 ml by rotary evaporations at 35°C. The purified metabolite was rechromatographed by HPLC using Method 1. The radiochromatogram revealed only one radioactive peak eluting at 6 min, while the uv chromatogram showed multiple peaks eluting around 6 min. This sample was analyzed by FAB LC/MS.

Synthesis of glutathione conjugate (STG). The commercially available disulfide of glutathione (GSH) GSSG was converted to bis-*N*-t-Boc-GSSG and then reduced to *N*-t-Boc-GSH via the procedure outlined in the literature (10). This material (0.25 g) was mixed with 1.1 equivalents of *N*-4-dimethylamino-6-(2,2,2-trifluoroethoxy)-1,3,5-triazin-2-yl-*O*-phenylcarbamate (6) in acetonitrile solution and then treated with 3.1 equivalents of triethylamine at 10°C followed by 4 hr at ambient temperature. This mixture was then diluted with water and four equivalents of $NaHCO_3$ and washed with ethyl acetate. The aqueous layer was acidified to pH 1 with 1 *N* HCl and extracted three times with ethyl acetate. The organic layer was dried over sodium sulfate and concentrated under reduced pressure to a white solid, m.p. 145°C (dec). The *N*-t-Boc group was then removed by dissolving a 50-mg portion of this product in 8 ml of a 1/1 mixture of trifluoroacetic acid and methylene chloride at ambient temperature for 30 min. Removal of the solvent *in vacuo* left an amorphous solid, m.p. 85–90°C (dec). The reactions were followed by TLC on silica gel using 7/2/1 ethyl acetate/methanol/water as the eluant. The synthetic conjugate *S*-[[*N*-4-dimethylamino-6-(2,2,2-trifluoroethoxy)-1,3,5-triazin-2-yl]carbamoyl]-glutathione (STG) exhibited the following spectral properties: NMR ^1H (DMSO-d_6) 2.0 (m,2), 2.32 (m,2), 2.95 (m,1), 3.04 (s,3), 3.11 (s,3), 3.25 (dd,1), 3.75 (m,3), 4.45 (m,1), 4.98 (q,2), and 8.3 (m, NH's) ppm; mass spectrum (FAB) *m/z* (% rel intensity) 571 (100, MH^+), 527 (5), 308 (15), 264 (98), and 238 (42).

RESULTS AND DISCUSSION

Selectivity studies. The site of action of triflusulfuron methyl is ALS. Determining if ALS sensitivity had a role in tolerance, crude enzyme extracts were obtained from the five species and tested *in vitro* for sensitivity to triflusulfuron methyl (Table 1). Although there were some differences in I_{50} values among the enzymes, these were small compared to the changes in plant tolerance. Moreover, the differences were not correlated with plant sensitivities. Sugar beet ALS had one of the lowest I_{50} values (33 n*M*), yet it is tolerant, while *B. napus* ALS had one of the highest I_{50} values (62 n*M*) and it is the most sensitive species. The high I_{50} value for *C. album* ALS might partially account for its moderate tolerance, but certainly cannot account for the degree of tolerance observed.

Uptake of triflusulfuron methyl following foliar application was low (Fig. 1) but within the range observed for thifensulfuron methyl (11). Although there were differences in uptake among the five plant species, there was no apparent correlation between uptake (as a percentage of applied herbicide) and plant sensitivity. Sugar beet had the lowest uptake, which could help to enhance selectivity, but *B. napus*, a sensitive species, had nearly identical low uptake values. Moreover, the moderately tolerate *C. album* and sensitive *V. persica*

TABLE 1
Inhibition of Plant Acetolactate Synthase by Triflusulfuron Methyl

Plant species	ALS I_{50} (n*M*)[a]	Plant response[b]
Sugar beet	33 ± 5	Tolerant
Chenopodium album	92 ± 16	Mod tolerant
Matricaria inodora	37 ± 5	Sensitive
Brassica napus	62 ± 11	Sensitive
Veronica persica	26 ± 4	Sensitive

[a] The concentration of triflusulfuron methyl required to inhibit ALS activity by 50% in a 30-min fixed time assay at 30°C. Values are means ± SE of two experiments, each with triplicate determinations.

[b] Based on whole plant greenhouse data.

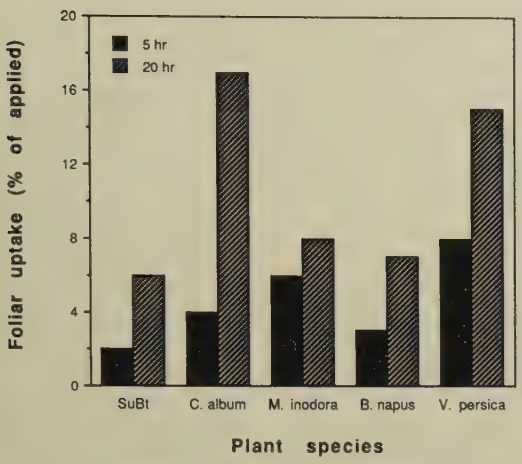


FIG. 1. *Uptake of [triazine-^{14}C]triflusulfuron methyl 5 and 20 hr after a foliar application (SuBt, sugar beet). The mean of the SD values for this experiment was 31%.*

species both had higher but similar uptake values. These results discount differential uptake as the basis for sugar beet tolerance to triflusulfuron methyl.

Figure 2 compares the metabolism of a transpired pulse of [^{14}C]triflusulfuron methyl by sugar beets and the four weed species. Sugar beets in the three- to four-leaf stage metabolized triflusulfuron methyl rapidly, with a half-life of less than 1 hr. The sensitive weeds *B. napus, V. persica,* and *M. inodora* metabolized triflusulfuron methyl much more slowly, resulting in half-lives of greater than 35 hr. *C. album* exhibited an

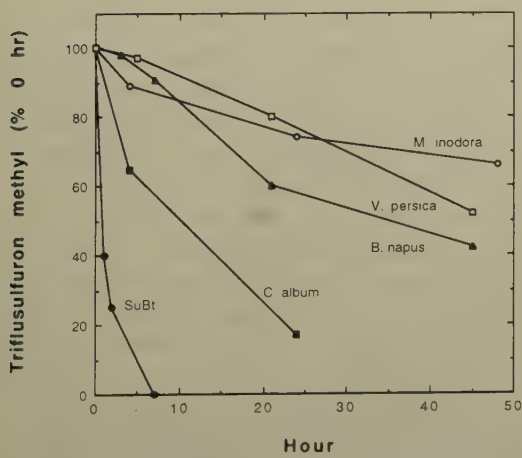


FIG. 2. *Metabolism of a transpired pulse of [triazine-^{14}C]triflusulfuron methyl by shoot explants.*

intermediate half-life (7 hr) consistent with its moderate tolerance to triflusulfuron methyl. The ^{14}C metabolic profiles (data not shown) showed only 3 to 5 metabolites for sugar beet and the sensitive weeds; however, the metabolites were not all the same for any two species. *C. album,* on the other hand, showed about 10 metabolites, suggesting more than one metabolic pathway.

Table 2 shows the combined results for the plant response (GR_{50}), foliar uptake, enzyme sensitivity (I_{50}), and metabolism studies. The only data that correlates with plant sensitivity are the rates of metabolism (inverse correlation with metabolic half-life). Furthermore, this rapid rate of metabolism in sugar beets is not restricted to the shoot, as experiments with roots indicated the same rapid metabolism (half-life ~1 hr) as was observed with the shoots (data not shown). These results indicate that tolerance in sugar beets is due to rapid metabolic inactivation of triflusulfuron methyl, which is the same mechanism for tolerance reported for other sulfonylurea herbicides (11–16).

Sugar beet metabolism studies. The rapid metabolism of triflusulfuron methyl by sugar beets cannot be explained by simple hydrolysis. Triflusulfuron methyl has a half-life of 3.7 days in water at pH 5 and 25°C, and the half-life increases at higher pH values resulting in values of 32 and 36 days at pH 7 and 9, respectively (1). In addition, the rate of hydrolysis declines at lower temperatures with a Q_{10} of about 2 (data not shown). Thus, the rapid metabolism by sugar beets is almost certainly enzyme catalyzed.

The initial metabolites observed in sugar beets (roots and leaves) for both the [*triazine-*^{14}C]- and [*ester carbonyl-*^{14}C]triflusulfuron methyl with extraction Method 2 are shown in Fig. 3. Only one metabolite peak was observed with the ester carbonyl label, but this peak actually represents both saccharin and its free acid, which could not be separated using HPLC Method 1. Metabolism of [*triazine-*^{14}C]triflusulfuron

TABLE 2
Triflusulfuron Methyl Uptake, Metabolism, and ALS I_{50} Values for Plants Differing in Sensitivity

Plant species	Plant sensitivity GR_{50} (g/ha)[a]	Enzyme sensitivity I_{50} (n*M*)	% Uptake after 20 hr	Metabolism half-life (hr)[b]
Sugar beet	>500	33	6	<1
C. album	110	92	17	7
M. inodora	17	37	8	80
B. napus	10	62	7	36
V. persica	25	26	15	61

[a] The rate of application required to inhibit plant growth 50% in the greenhouse. Values are the means of at least six different experiments.

[b] Values calculated from the data in Fig. 2. The SE of the mean averaged 15% for these values.

methyl in leaves yielded the triazine amine and a more polar 6-min metabolite. The 6-min metabolite did not appear in chromatograms when leaves were extracted with acetone/ammonium carbonate (extraction Method 1) rather than acetone/water. Instead, the triazine urea was observed, suggesting that the 6-min metabolite might be the triazine isocyanate and would react with ammonia to form the triazine urea. Furthermore, we found that the 6-min metabolite degraded to the triazine amine under acidic conditions, again suggesting the isocyanate. On the other hand, a highly reactive triazine isocyanate would not be expected to be formed or exist long in an aqueous environment, and the metabolite's short retention time suggested a substance with a much higher polarity than would be expected for this isocyanate. LC/MS (FAB) of the isolated 6-min metabolite yielded a strong protonated molecule (MH^+) ion of 571 and fragment ions at *m/z* 308, 264, and 238 (Fig. 4). The 571 ion is indicative of the glutathione conjugate STG, which has a MW of 570. This identification was confirmed by the synthesis of STG and comparison of the mass spectra and HPLC retention times with the 6-min metabolite. The glutathione carbamate STG would be expected to exhibit reactions similar to the triazine isocyanate as discussed above. Although glutathione is known to have a role in the metabolism and detoxification of many xenobiotics in plants (17), this is the first report that we are aware of that shows its involvement in sugar beets.

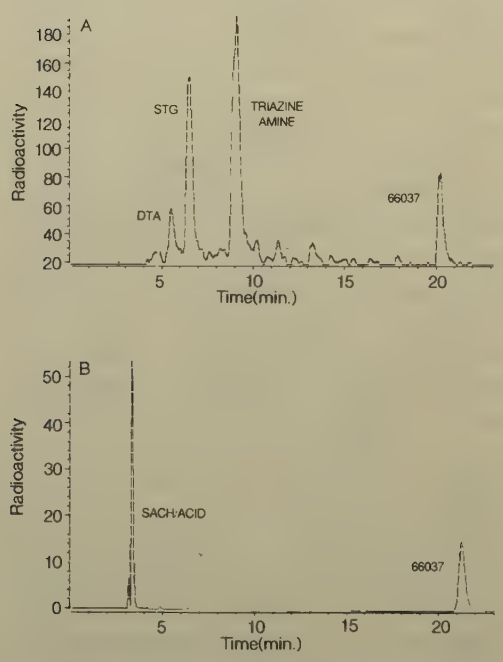


FIG. 3. *Reverse-phase HPLC analysis of* (*A*) [*triazine-*^{14}*C*]- *and* (*B*) [*ester carbonyl-*^{14}*C*]*triflusulfuron methyl and its metabolites extracted from sugar beet leaves 2 hr after treatment. DTA, N-desmethyl triazine amine; STG, S-(N-triazinyl-carbamoyl)glutathione; SACH, methylsaccharin; ACID, acid sulfonamide; 66037, triflusulfuron methyl.*

A proposed pathway for the initial metabolism of triflusulfuron methyl in sugar beet is shown in Fig. 5. Attack of the glutathione sulfhydryl group on the urea carbonyl of triflusulfuron methyl gives rise to the tetrahedral intermediate shown in Fig. 5 (bracketed structure). This can subsequently revert to sulfonylurea and GSH or collapse to afford the observed *S*-(*N*-

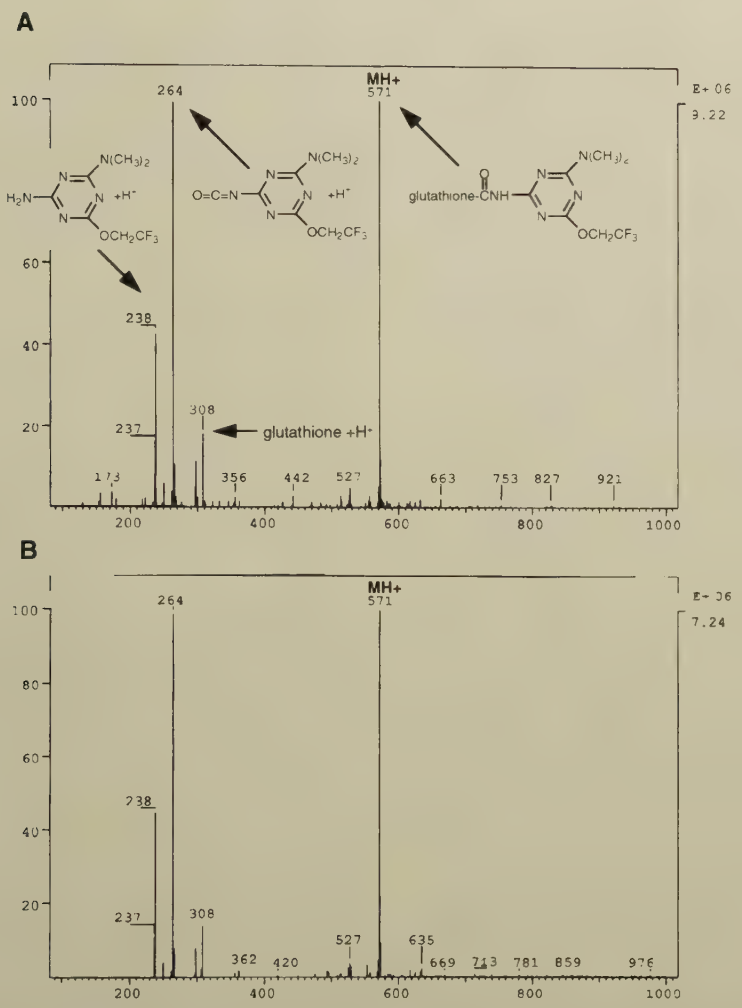


FIG. 4. *Mass spectra of (A) the isolated 6-min metabolite and (B) the synthesized glutathione conjugate of the isocyanate.*

triazinyl-carbamoyl) GSH (STG), 7-methylsaccharin, and methanol. The congested 2,6-disubstituted moiety in triflusulfuron methyl makes conversion to the saccharin more thermodynamically favorable than in ester sulfonylureas without this substitution pattern. Relief of the added steric crowding caused by the 6-methyl group provides the driving force for this reaction and may be the basis for sugar beet tolerance to this herbicide. This conjugate is somewhat reactive as well and would be susceptible to attack by other nucleophiles such as ammonia when exposed to this during extraction. Cleavage of triflusulfuron methyl to the triazine amine plus 7-methylsaccharin and its free acid may also occur via aqueous hydrolysis.

Structure-selectivity studies. The effect of changes in structure on selectivity is shown in Table 3. These compounds are all highly active against ALS as demonstrated by their herbicidal activity on sugar beet and/or rape. Yet, small changes in structure result in dramatic changes in selectivity. The importance of the carboxylic ester at the 2-position and the methyl at the 6-position of the ring for sugar beet selectivity is

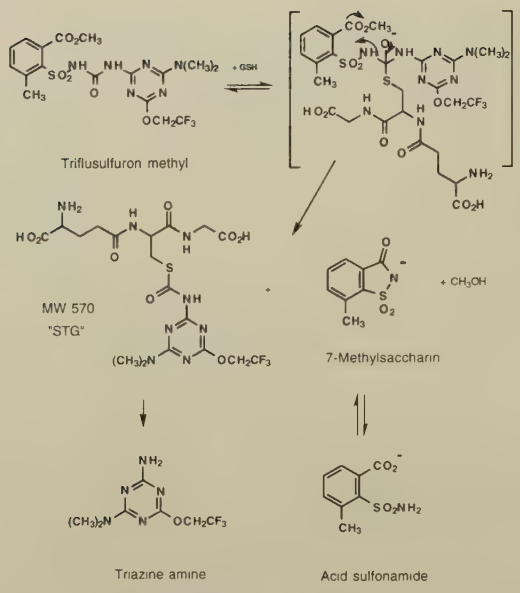


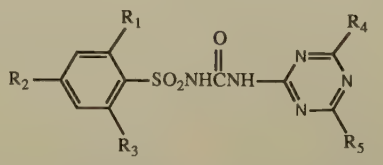
FIG. 5. *Proposed pathway for the initial metabolism of triflusulfuron methyl in sugar beets.*

evident. As discussed above, the steric crowding caused by the methyl group in the 6-position may play a role in enhancing the conjugation reaction with GSH. It appears, however, that the methyl group must have importance beyond size since replacement with a similar size group, e.g., Cl, does not result in sugar beet selectivity. Moreover, minor changes on the triazine ring (replacing trifluoromethyl with methyl and removing one of the methyl groups on the amine) can also abolish sugar beet selectivity. Furthermore, combining these minor changes on the triazine ring with removal of the 6-methyl group on the phenyl ring reverses selectivity, so rape is now tolerant and sugar beet is the sensitive species. The fact that these minor changes in structure can have such a dramatic effect on selectivity further supports the role for an enzyme in sugar beet metabolism of triflusulfuron methyl.

The enzyme most likely to catalyze the formation of the glutathione conjugate STG from triflusulfuron methyl would be GST. Yet, attempts to use an inhibitor (tridiphane) to obtain further evidence for the involvement of GST proved inconclusive. Tridiphane had no effect on the rate of me-

TABLE 3

Minor Changes in the Structure of Triflusulfuron Methyl Markedly Affect Herbicidal Selectivity



R_1	R_2	R_3	R_4	R_5	GR_{50} Beet	GR_{50} Rape
$COOCH_3$	H	CH_3	$N(CH_3)_2$	OCH_2CF_3	>500	10
$COOC_3H_7$	H	CH_3	$N(CH_3)_2$	OCH_2CF_3	>500	3
$COOCH_3$	CH_3	H	$N(CH_3)_2$	OCH_2CF_3	220	150
$COOCH_3$	H	H	$N(CH_3)_2$	OCH_2CF_3	1	1
$COOCH_3$	H	Cl	$N(CH_3)_2$	OCH_2CF_3	10	10
$COOCH_3$	H	CH_3	$NHCH_3$	OCH_2CF_3	180	28
$COOCH_3$	H	CH_3	$NHCH_3$	OCH_2CH_3	10	18
$COOCH_3$	H	H	$NHCH_3$	OCH_2CH_3	2	860

tabolism of triflusulfuron methyl in sugar beet leaves, but it is uncertain whether it even reached the site of action.

In summary, sugar beet tolerance to triflusulfuron methyl is due to rapid metabolism of the herbicide in both roots and leaves, resulting in a half-life of about 1 hr. This metabolism is initiated by nucleophilic attack by GSH at the urea carbonyl, producing the GSH conjugate STG, 7-methylsaccharin and its free acid, which are all herbicidally inactive. These results support and extend previous studies, which have established that metabolic inactivation is the basis for selectivity of sulfonylurea herbicides on crop plants (11–16).

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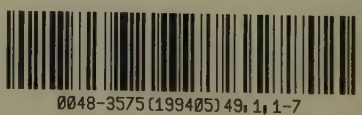

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Neurophysiological Effects and Quantitative Structure–Activity Analyses of Insecticidal Silaneophanes[1]

HIROSHI OKIMOTO, KEIICHIRO NISHIMURA,[2] KAZUHIKO MATSUDA,* MASAYUKI HAMADA,* TAMIO UENO, AND TOSHIO FUJITA

*Department of Agricultural Chemistry, Kyoto University, Kyoto 606-01, Japan; and *Department of Agricultural Chemistry, Kinki University, Nara 631, Japan*

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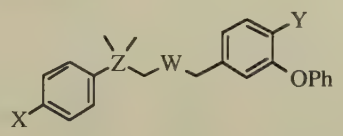
Silaneophanes containing a quaternary silicon connected to variously substituted phenyl groups were shown to prolong the sodium residual and tail currents in the crayfish giant axon under voltage clamp conditions, sharing characteristics common to conventional pyrethroids. Insecticidal activity of these compounds was measured against American cockroaches. Variations in the insecticidal activity measured under conditions to inhibit the oxidative metabolic mechanism were analyzed quantitatively. It was found that insecticidal activity was linearly related to the crayfish tail-current prolongation activity when the transport factors of compounds were allowed for by use of a hydrophobicity parameter. The higher the tail-current prolongation activity, the higher the insecticidal activity. Variations in the insecticidal activity were also correlated with physicochemical substituent parameters, indicating that the longer the substituents on the benzene ring, the higher the activity. There was an optimum in the hydrophobicity of the substituents, probably related to factors involved in the transport process. © 1994 Academic Press, Inc.

INTRODUCTION

One of the limitations to the use of conventional synthetic pyrethroids having a high insecticidal activity in paddy fields is that they generally have a high toxicity to fishes (1). Thus, structure modification of synthetic pyrethroids has been attempted toward new types of insecticides hopefully devoid of fish toxicity. Etofenprox (I: W = O, X = OEt, Y = H, Z = C) and MTI-800 (I: W = CH_2, X = OEt, Y = F, Z = C) belong to such a new class of compounds that have no ester bonding. However, they still show a certain level of fish toxicity, although it is markedly reduced from that of the conventional pyrethroids (2). With further development of this chemistry, quaternary silicon analogs with structure I have been synthesized (1, 3–9). Among them, silafluofen (I: W = CH_2, X = OEt, Y = F, Z = Si) showed high insecticidal activity but only a negligibly low fish toxicity and is expected to be an excellent candidate insecticide in paddy fields (1, 10).



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We previously showed that etofenprox and related compounds modify the sodium current in crayfish axonal membranes under voltage clamp conditions similar to conventional pyrethroids (11). Whereas certain differences have been shown to exist in the biochemical and pharmacokinetic effects between silafluofen and conventional pyrethroids (12), silafluofen and related compounds have been indicated to show neuroexcitatory profiles in cockroach nerve preparations similar to those exhibited by etofenprox (13). The chemical structure of silafluofen is very close to that of MTI-800. The neurophysiological mode of action of silafluofen and analogs could therefore be reasonably close to that of etofenprox and MTI-800 and, in turn, to that of conventional pyrethroids.

[1] Quantitative Structure–Activity Studies of Pyrethroids, Part 30.

[2] To whom correspondence should be addressed.

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In this study, we examined the neurophysiological effects of silaneophanes (I: $W = CH_2$, $Y = H$, $Z = Si$) with various substituents X. We found that this series of compounds prolongs the sodium currents to various extents in the crayfish axonal membranes under voltage clamp conditions similar to etofenprox. We also analyzed quantitatively the variations in the insecticidal activity against American cockroaches with an index for the sodium-current prolongation activity as well as with physicochemical substituent parameters of X. Based on comparisons of these results with those reported previously for various conventional pyrethroids, we concluded that the insecticidal activity of this series of compounds is primarily due to their effect on sodium channels of the nerve membrane sharing a common neurophysiological mode of action with etofenprox and pyrethroids.

MATERIALS AND METHODS

Compounds

Table 1 shows the test compounds used in this study. They were generous gifts from Hoechst AG, Germany. Piperonyl butoxide (PB)[3] was used as an inhibitor of oxidative metabolism. In some experiments, NIA16388 (NIA) also was used as an inhibitor of hydrolytic metabolism (14). Test compounds were dissolved in methanol (MeOH) for the measurement of the insecticidal activity and in dimethyl sulfoxide (DMSO) for the voltage clamp experiments. The inhibitors of metabolism were dissolved in MeOH. All solutions were stored at −20°C until use.

Measurement of the Sodium Current under Voltage Clamp Conditions

Voltage clamp experiments were performed with giant axons of crayfish, *Procambarus clarkii,* by use of the double sucrose-gap technique according to a procedure described previously (11). The sodium current was measured after eliminating the potassium current by internal perfusion of the axon with a solution in which potassium ions in the standard solution were substituted by cesium ions (15). The holding membrane potential was −100 mV. The ohmic leakage and capacitive currents associated with step changes in the membrane potential were estimated by extrapolation of the currents associated with step hyperpolarization to −140, −130, and −120 mV and were subtracted from the currents observed. Membrane currents induced by a step depolarizing pulse were digitized and stored on Hewlett–Packard 9121 D/S flexible disk memory or TEAC CT-500 magnetic tape. Test solutions were prepared by addition of a small portion of the DMSO solution of test compounds into the potassium-free internal saline. Some solutions were turbid because of their poor solubility, but were used as such. In these cases, the concentration of compounds was taken to be that as if they were completely dissolved. The final concentration of DMSO in the internal saline was lower than 0.5% (v/v). DMSO alone in this concentration had no effect on the sodium currents. Measurements were carried out at 6 ± 2°C.

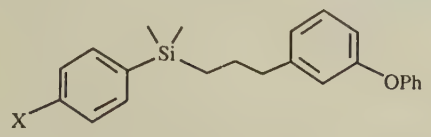
Measurement of Insecticidal Activity against American Cockroaches

The insecticidal activity of the compounds was measured against male adult American cockroaches, *Periplaneta americana,* by a procedure described previously (14). Unless otherwise noted, a MeOH solution (1 μl) containing 50 μg of PB was first injected into the abdomen of the insect. One hour later, various amounts of each compound as the MeOH solution (1 ~ 10 μl) were injected into the abdomen of the insects. After being left at 20 ± 1°C for 24 hr, the symptoms of the insects were observed. Unless otherwise noted, NIA was not used as the metabolic inhibitor. NIA did not affect much the insecticidal activity

[3] Abbreviations used: PB, piperonyl butoxide; NIA, NIA16388 or propargyl propyl benzenephosphonate; MeOH, methanol; DMSO, dimethyl sulfoxide; MLD, minimum lethal dose.

TABLE 1

Biological Activities and Physicochemical Parameters of Silaneophane Derivatives



Compound number	X	log(1/MLD) Obs[a]	log(1/MLD) Calcd Eq. (6)	log(1/MLD) Calcd Eq. (10)	log τ	ΔL	π	log P
1	H	7.97	8.02	7.99	2.15	0.00	0.00	9.36
2	F	8.13	7.97	8.12	2.10	0.59	0.14	9.50
3	Cl	8.02	8.07	8.19	2.32	1.46	0.71	10.07
4	Br	8.11	8.08	8.19	2.37	1.76	0.86	10.22
5	Me	8.29	8.21	8.09	2.33	0.81	0.56	9.92
6	Et	7.86	8.03	8.18	2.39	2.05	1.02	10.38
7	Bu(*t*)	<6.88	7.55	7.46	2.44	2.05	1.98	11.34
8	CF_3	8.20	8.34	8.07	2.66	1.24	0.88	10.24
9	CHO	8.22	8.41	8.21	2.65	1.47	−0.65	8.71
10	OMe	8.53	8.45	8.41	2.61	1.92	−0.02	9.34
11	OEt	9.02	8.81	8.56	3.03	2.74	0.38	9.74
12	OPr(*n*)	8.34	8.41	8.47	2.80	3.42	1.05	10.41
13	OPr(*i*)	8.52	8.65	8.41	2.98	2.74	0.85	10.21
14	OPh	<7.71	7.10	7.44	2.07	2.45	2.08	11.44
15	$OCHF_2$	8.76	8.65	8.39	2.85	1.92	0.31	9.67[b]
16	OCH_2CH_2F	8.73	8.65	8.68	2.83	3.17	0.18	9.54[c]
17	OCH_2CF_3	8.60	8.60	8.26	3.09	2.94	1.22	10.58[c]
18	SMe	8.16	8.20	8.39	2.43	2.24	0.61	9.97
19	SEt	7.89	7.92	8.38	2.29	3.10	1.07	10.43
20	$N(Me)_2$	7.87	7.91	8.31	2.04	1.47	0.18	9.54
21	$Si(Me)_3$	6.87	6.80	6.81	2.24	2.28	2.59	11.95

[a] Under conditions with PB.

[b] The value was taken from the data bank issued by the Pomona College Medicinal Chemistry Project (Claremont, CA).

[c] log *P* values calculated by use of the software developed by the project in *b*.

of this series of nonester compounds as shown later.

Three insects were used for the test of each dose of each compound. The minimum dose at which two of three insects were counted as killed was defined as minimum lethal dose (MLD, in moles). For the MLD measurement, three to five different doses were examined first to roughly estimate the "minimum" dose. After adjusting the intervals of log(dose) to ±0.1 around the minimum dose, the minimum dose was estimated repeatedly at least two times to give reproducible minimum log(dose) values. For each log(1/MLD) value, 30 to 40 insects were generally used altogether. Paralyzed insects were counted as those being killed. Table 1 shows the log(1/MLD) values for test compounds. Each value is the mean for at least two repetitions. The standard error of the mean log(1/MLD) value was within ±0.2.

Correlation Analysis

We examined the relationship between insecticidal and neurophysiological activities by using the equation

$$\log(1/\mathrm{MLD}) = a(\log \tau) + b(\log P) - c(\log P)^2 + \text{constant}, \quad [1]$$

where *a*, *b*, *c*, and the constant were evaluated by regression analysis, log τ is the index for the tail-current prolongation activity defined below, and log *P* represents

the hydrophobicity of the molecule, in which P is the partition coefficient in the 1-octanol/water partitioning system. The squared log P term was added to identify the optimum hydrophobic effect so that $c \geqq 0$.

Variations in the insecticidal activity were analyzed with physicochemical parameters of aromatic substituents X by

$$\log(1/\mathrm{MLD}) = a\Delta L - b(\Delta L)^2 + c\pi - d\pi^2 + \text{constant}, \quad [2]$$

where the ΔL is the STERIMOL maximum length of each substituent relative to that of H (16), the π is the hydrophobic substituent parameter derived from log P values of monosubstituted benzenes (17), and a, b, c, d, and the constant were calculated by regression analysis. Table 1 shows the substituent constants.

The P values in this series of compounds were expected to be very high. Thus, the log P value of the ethoxy compound **11** was first calculated from the log P values for reference compounds according to

$$\log P(\text{compound 11}) = \log P(\text{MTI-790}) - \log P[\text{Ph-C}(\text{CH}_3)_3] + \log P[\text{Ph-Si}(\text{CH}_3)_3], \quad [3]$$
$$9.74 \qquad 9.03 \qquad 4.12 \qquad 4.83$$

where the log P value for MTI-790 (I: W = CH_2, X = OEt, Y = H, Z = C) was taken from our previous paper (18), the log P values for other reference compounds were taken from the literature (13), and the log P values for other test compounds listed in Table 1 were calculated according to the additivity principle by use of the π values from the monosubstituted benzene system (19).

RESULTS

Effects on Sodium Currents under Voltage Clamp Conditions

Before application of the test compounds, the inward current in the crayfish giant axon in response to a −20 mV test pulse increased rapidly and then decreased to give a peak current (Fig. 1A). After application of 6.9×10^{-5} M compound **10**, the amplitude of the peak current was reduced markedly and the falling phase was slowed to induce a large residual current during the step depolarization (Fig. 1B). Upon step repolarization, a large and slowly decaying tail current was observed. It took a number of seconds until the tail current decayed to the zero level (Fig. 1C). Similar observations were made for other compounds, although the rate of the decay varied depending on the substituent X. The potassium ions were replaced by cesium ions in the internal saline; therefore, the currents observed were due to those through sodium channels.

To quantify the rate of decay, the tail current was redrawn on semilog coordinates (Fig. 2). The semilog plots represent a straight line, so the fall of the tail current obeys the first-order kinetics as shown in the equation,

$$\ln(-I_{\text{tail}}) = -t/\tau + \text{constant}, \quad [4]$$

where $-I_{\text{tail}}$ ($I_{\text{tail}} < 0$) is the inward tail current (in μA), t is the time (in msec) after the step repolarization of the membrane, and τ is the reciprocal of the rate constant of the decay. The higher the τ value, the slower the rate of decay; i.e., the higher the tail-current prolongation activity. The $1/\tau$ value for each compound was calculated by the least-squares method with Eq. [4].

The log τ value of a number of pyrethroids has been shown not to be changed by experimental conditions such as the duration and potential of test pulses or the concentration of compounds tested (20). We confirmed that the log τ value is "independent" of these experimental conditions also for compounds in this series using

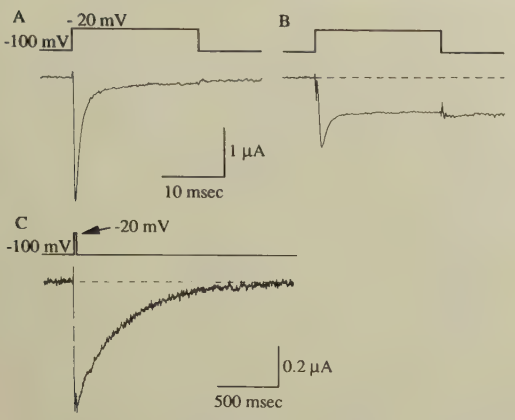


FIG. 1. *Sodium currents recorded from an internally perfused crayfish giant axon. The axon was step-depolarized from holding potential (−100 mv) to −20 mV for 20 msec before (A) and 4 hr after internal application of 6.9 × 10⁻⁵ M compound* **10** *(B, C). The dotted lines in B and C indicate the zero level in the current.*

compounds **8** and **11** (Table 2). The size of the tail current increased with time until a steady state was attained (21). The tail current was measured at the steady state to calculate the τ value. Although the initial size of the tail current was affected by the concentration of the compound, the log τ value was virtually independent of the concentration as shown in Table 3 for compound **8**. However, the larger the initial size of the tail current, the more accurately estimable the log τ value. Therefore, we selected appropriate conditions to induce a large tail current. The log τ values listed in Table 1 are the mean of values estimated from various concentrations between 3.0×10^{-6} and 1.3×10^{-4} *M* with depolarizing

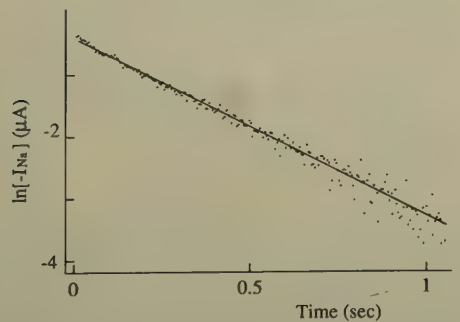


FIG. 2. *Semilog plot of the tail current against time. Original data were taken from Fig. 1C. The straight line was obtained by regression analysis.*

TABLE 2
Effect of Pulse Conditions on log τ Value

Compound **8**[a]			Compound **11**[a]		
Duration (msec)	Potential (mV)		Duration (msec)	Potential (mV)	
	−20	20		−20	20
20	2.50	2.52	20	3.03	3.03
50	2.55	2.52	50	3.05	3.05
100	2.55	2.56	100	3.04	3.05
200	2.58	2.57	200	3.05	3.05

[a] Tested at 3.0×10^{-6} *M*.

pulses at −20, 0, and 20 mV and different pulse durations of 10–200 msec. Two or more axonal preparations were used for most compounds. The standard error of the mean value ($n = 3 \sim 15$) was less than ±0.2.

Insecticidal Activity against American Cockroaches

Table 4 shows the insecticidal activity of some compounds measured under various synergistic conditions. PB enhanced the activity by 0.2–0.5 log unit. The enhancement in an ester-type pyrethroid, phenothrin, by PB was also in this range. Further addition of NIA did not increase the activity of compounds **1**, **11**, and **15** as prominently as the activity of phenothrin that is increased by about 0.5 log units. The activity of phenothrin measured with both synergists was slightly higher than that of silafluofen measured in the presence of PB.

Because the effect of NIA on silaneophanes was negligible, the "intrinsic" insecticidal activity was taken as that under the synergistic conditions with PB alone. The activity of the ethoxy derivative (com-

TABLE 3
Effect of the Concentration of Compound 8 on log τ Value

Concn (*M*)	log τ[a]
3.0×10^{-6}	2.55
1.0×10^{-5}	2.74
8.6×10^{-5}	2.69[b]

[a] Axonal membrane was step-depolarized by a pulse with 100 msec duration at −20 mV.
[b] The solution was turbid.

TABLE 4
Effect of Synergists on Insecticidal Activity

Compound[a]	log(1/MLD) Alone	+PB	+(PB + NIA)
1	7.50	7.97	7.95
11	8.81	9.02	9.11
15	8.51	8.76	8.81
Silafluofen	8.99	9.29	—[b]
Phenothrin	8.83[c]	9.04[c]	9.49[c]

[a] Compound number corresponds to that in Table 1.
[b] Not measured.
[c] Data from Ref. (14).

TABLE 5
Insecticidal and Sodium-Current Prolongation Activities of Conventional Pyrethroids, MTI Compounds, and Silafluofen and Other Silaneophane Derivatives

	log(1/MLD)[a]	log τ[b]
Esfenvalerate[c]	10.65	5.58
Deltamethrin[d]	10.94	5.11
Cyphenothrin[d]	10.71	5.10
MTI-800	10.16	4.12
Phenothrin[e]	9.49	3.42
Tetramethrin[e]	8.42	3.21
Etofenprox	9.71	2.97
Silafluofen	9.29	3.44
Compound 11	9.02	3.03
Compound 17	8.60	3.09

[a] Under conditions eliminating metabolic degradation as far as possible. From Ref. (22), except for silicon compounds.
[b] Except for silicon compounds, values were cited from Ref. (22).
[c] The *S*-alcohol ester of the *S*-acid.
[d] The 1*R*(*trans*)-acid ester of the (*RS*)-alcohol.
[e] The 1*R*(*trans*)-acid ester.

pound 11) was the highest, followed by the monofluoro (compound 16) and trifluoroethoxy (compound 17) derivatives. That of the trimethylsilyl derivative (compound 21) was the lowest among compounds with which definitive log(1/MLD) values were measurable (Table 1). The MLD values of compounds 7 and 14 were not measurable within the limit of solubility in MeOH.

DISCUSSION

The insecticidal activity of tested silaneophane derivatives was in the range of that of conventional pyrethroids measured previously (22) as shown in Table 5. It seems to vary roughly in parallel with the tail-current prolongation activity among compounds including pyrethroids and silaneophane derivatives together. This trend was also observed in Table 1 for the silaneophanes alone. Some alkoxy compounds, 11, 13, and 15–17, are insecticidally as well as neurophysiologically more active than others, whereas the reverse is the case for compounds 1, 2, 14, and 19–21. The variations in the tail-current activity, log τ, seemed not to be exactly but approximately linear with those in the insecticidal activity, log(1/MLD), as shown in Fig. 3. The situation is formulated as Eqs. [5]–[8] with the use of Eq. [1].

By the single-term analysis, the equation

$$\log(1/\mathrm{MLD}) = \underset{(1.249)}{5.565} + \underset{(0.489)}{1.046}(\log\tau)$$
$$n = 19,\ s = 0.321,\ r = 0.738,\ F(1,17) = 20.3 \quad [5]$$

was obtained. In this and the following equations, n is the number of compounds, s is the standard deviation, r is the correlation coefficient, and F is the ratio between regression and residual variances. Addition of quadratic hydrophobicity terms to Eq. [5] improved the correlation to give

$$\log(1/\mathrm{MLD}) = \underset{(8.637)}{-10.648} + \underset{(0.202)}{0.939}(\log\tau) + \underset{(1.694)}{3.567}(\log P) - \underset{(0.082)}{0.191}(\log P)^2$$
$$n = 19,\ s = 0.129,\ r = 0.967,\ F(3,15) = 72.7. \quad [6]$$

Whereas the insecticidal effect is caused by interactions from "outside" the nervous system, the tail-current activity was measured in the axon in which compounds were applied internally. Nevertheless, the tail-current activity could be used as a param-

eter representing the nerve activity to correlate with the insecticidal potency. Insecticides may act after being incorporated inside the axon. Equation [6] means that the higher the tail-current prolongation activity, the higher the insecticidal activity. There is an optimum in the hydrophobicity that reflects the optimum transport conditions from the site of injection to the target site in the insect body at about log P = 9.3. Table 1 shows the log(1/MLD) values calculated by Eq. [6]. The inactivity of the phenoxy derivative (compound **14**) is well predicted by Eq. [6], but that of the *tert*-Bu derivative (compound **7**) is not. However, the calculated values of these two compounds are among the lowest, which could conform with the observed inactivity at least in part.

Our previous analyses of the cockroach insecticidal activity with the sodium tail-current activity in crayfish giant axons for various conventional pyrethroids gave (23)

$$\log(1/\mathrm{MLD}) = \underset{(0.596)}{4.926} + \underset{(0.142)}{0.862}(\log\tau) + \underset{(0.097)}{0.155}(\log P)$$
$$n = 64,\ s = 0.419,\ r = 0.857,\ F(3,60) = 56.6. \quad [7]$$

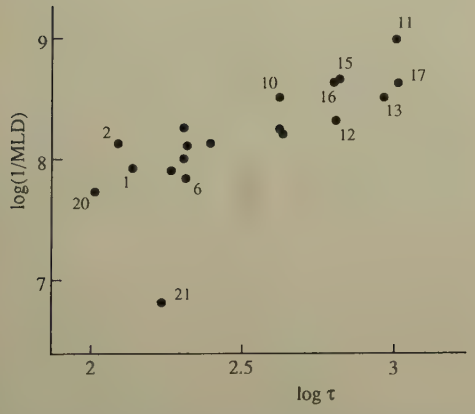
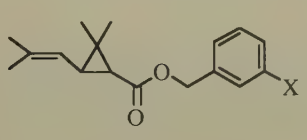


FIG. 3. *Relationship between the insecticidal and tail-current activities of silaneophanes. The number of compounds corresponds to that in Table 1.*

The slope of the log τ term in Eq. [7] is close to that in Eq. [6]. Therefore, the compounds were combined to give

$$\log(1/\mathrm{MLD}) = \underset{(1.062)}{3.560} + \underset{(0.126)}{0.882}(\log\tau) + \underset{(0.302)}{0.611}(\log P) - \underset{(0.020)}{0.037}(\log P)^2$$
$$n = 83,\ s = 0.384,\ r = 0.880,\ F(3,79) = 90.1. \quad [8]$$

Equation [8] indicates that variations in the insecticidal activity of compounds including various types of conventional pyrethroids and silaneophanes are closely connected with the tail-current activity on the same background, even though the routes of application and organisms used to estimate the biological activity parameters are different. The fact that the slope of the log τ term is close to unity would indicate that, after separating the transport factors, the variations in the insecticidal activity would primarily be governed by the variations in the current-modifying effect that is shared by conventional pyrethroids and silaneophanes. The relatively low importance of the log P term in Eq. [7] is probably due to the fact that the log P value of the compounds included does not increase sufficiently above the optimum value. However, the log P value of compounds included in Eq. [6] is mostly above the optimum. Thus, log P and $(\log P)^2$ terms in Eq. [8] might be more reliable than those in Eqs. [6] and [7]. However, the quality of the correlation of Eq. [8] is not as good as that of Eq. [6]. For variations in the insecticidal activity of a variety of structural series of compounds, factors other than the tail-current potency and molecular hydrophobicity seem to be involved, but are not well separated, leading to some distortion of the correlation. In fact, if we select such a uniform set of compounds as *meta*-substituted benzyl chrysanthemates (II) in-



II

cluding phenothrin (X = OPh) for the same type of analysis, we can formulate (23)

$$\log (1/\text{MLD}) = \underset{(7.549)}{-6.868} + \underset{(0.179)}{1.074}(\log \pi) + \underset{(2.362)}{3.607}(\log P) - \underset{(0.184)}{0.261}(\log P)^2$$
$$n = 29,\ s = 0.291,\ r = 0.955,\ F(3,25) = 86.9. \qquad [9]$$

Equation [9] is very similar to Eq. [6] except for the intercept. Thus, factors not separated well, which are probably attributable to characteristics of the mother skeletal structures, could be reflected in the difference in the intercept value between Eqs. [6] and [9]. The quadratic log P terms in Eqs. [6] and [9] could supersede those in Eq. [8].

Variations in the insecticidal activity of the compounds were analyzed by use of Eq. [2] with the physicochemical parameters of the aromatic substituents to give

$$\log (1/\text{MLD}) = \underset{(0.318)}{7.990} + \underset{(0.151)}{0.220}\Delta L - \underset{(0.094)}{0.251}\pi^2$$
$$n = 19,\ s = 0.273,\ r = 0.830,\ F(2,16) = 17.9. \qquad [10]$$

Addition of the $(\Delta L)^2$ term did not improve Eq. [10]. Neither additions of nor replacements with such steric parameter terms as the STERIMOL width and van der Waals volume gave any significant improvement. Equation [10] shows that the insecticidal activity varies parabolically with the hydrophobicity of substituents, an optimum π value being around zero, corresponding to an optimum log P of about 9.4. The positive sign of the ΔL term indicates that the longer the substituents, the higher the activity. Tables 6 and 7 show the development of this equation and the intercorrelation of variables, respectively. Table 1 also shows the log(1/MLD) values calculated by Eq. [10].

The counterpart of Eq. [10] with log P and $(\log P)^2$ terms was given by

$$\log (1/\text{MLD}) = \underset{(19.132)}{-9.862} + \underset{(0.167)}{0.235}\Delta L + \underset{(3.713)}{3.917}(\log P) - \underset{(0.179)}{0.215}(\log P)^2$$
$$n = 19,\ s = 0.280,\ r = 0.834,\ F(3,15) = 11.4. \qquad [11]$$

It is interesting to note that the log P and $(\log P)^2$ terms are very similar to those in Eq. [6], suggesting that, at least for this set of uniform compounds, these quadratic terms represent almost entirely hydrophobic factors participating in the transport process but not in the receptor binding.

We previously analyzed variations in the insecticidal activity against American cockroaches with physicochemical molecular and substituent parameters of the acid moiety of synthetic pyrethroids III and IV (24). These compounds share a common *meta*-phenoxybenzyl substructure in the "alcohol" moiety with the present series of compounds. In structure III, R_1 is the *iso*-propyl or *tert*-butyl group, and R_2 is one of

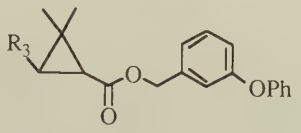
TABLE 6
Development of QSAR of Eq. [10]

Intercept	π^2	ΔL	s	r	$F(X,Y)^a$
8.396	−0.220		0.335	0.710	$F(1,17) = 17.30$
8.991	−0.252	0.219	0.273	0.832	$F(1,16) = 32.49$

[a] F statistic for significance of the addition of variables. X, the number of independent variables added at each step of the development. Y, $n - m - 1$, n being the number of data points and m being the total number of independent variables in the developed equation. Theoretical F values: $F(1,17,0.05) = 4.45$, $F(1,16,0.05) = 4.49$.

TABLE 7
Squared Correlation (r^2) Matrix for Variables Used to Derive Eq. [10]

	ΔL	π	π^2
π	0.151	1.000	
π^2	0.053	0.713	1.000



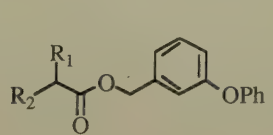
IV

alkoxy, aryl, aryloxy, and arylamino groups. R_3 in structure IV is one of variously substituted vinyl groups. We have found slight errors in the previous correlation equations that do not seriously distort previous conclusions. Recalculation of the correlations gave, as the best correlation for these compounds, the equation

$$\begin{aligned}\log(1/\mathrm{MLD}) = {} & -3.951 + 1.104\Delta L - 0.089(\Delta L)^2 + 2.184(\log P) \\ & (5.190)\quad (0.335)\qquad (0.034)\qquad (1.482) \\ & -0.142\,(\log P)^2 + 0.258\Delta(\Delta E) - 0.341\Delta n_{\mathrm{DOF}} \\ & (0.103)\qquad\quad (0.065)\qquad\quad (0.103) \\ n = {} & 80,\ s = 0.490,\ r = 0.876,\ F(6,73) = 40.0. \end{aligned} \qquad [12]$$

In Eq. [12], ΔL is the length parameter for the acid moiety to a specified direction similar to that of ΔL in Eq. [10]. $\Delta(\Delta E)$ and Δn_{DOF} are empirical parameters for the receptor binding enthalpy and entropy, respectively. We would like to point out here that, despite significant contributions from the receptor binding parameters, the structural elements in the "acid" moiety contributing to the variations in insecticidal activity are similar to those in the phenylsilanyl moiety of the silafluofen analogs. The optimum log P for compounds III and IV is about 7.7, which is not very far apart from 9.1~9.4 in Eqs. [10] and [11]. The standard used to calculate ΔL in compounds included in Eqs. [10] and [11] is somewhat different from that used for compounds in Eq. [12]. However, the range of the variations in ΔL value in Eqs. [10] and [11] is much narrower (3.42) than that in Eq. [12] (6.89), resulting in no significance of the $(\Delta L)^2$ term in Eqs. [10] and [11].

The trialkylsilyl group on the benzene ring is more electron-donative than tertiary alkyl substituents because of the delocalization of d-electron pairs through a d–π conjugation. However, unless X-substituents are electron-withdrawing mesomerically, no specific electronic interaction is significant between X and Si in silaneophanes and the regular σ or σ^0 could represent the electronic effect of X. Sieburth and co-workers (25) have recently analyzed the insecticidal activity of a comparable series of compounds against southern army worms with substituent parameters. They claimed that the steric bulk in terms of molar refractivity and electron-donating property in terms of negative σ of X-substituents are the parameters to rationalize the insecticidal potency. The addition of the σ term or the use of the chemical shift value in the ^{13}C NMR of the carbon atom in Me–Si and C (Ar)–Si bonds as the electronic parameter in Eqs. [10] and [11] did not improve the correlation at all. Because Sieburth and coworkers used only six compounds, we believe that their correlation may not be very reliable and no significant electronic effect of X-substituents is working to govern the variations in the insecticidal activity in general.



III

In summary, the high insecticidal activity of silaneophanes was correlated with their neurophysiological effect to prolong the open state of the sodium channels on the nerve membrane after receiving stimuli. This effect was similar to the effect caused by non-ester-type as well as conventional pyrethroids. The quantitative analyses for correlations between insecticidal and neurophysiological activities and between insecticidal activity and physicochemical molecular parameters were similar to those previously found for conventional pyrethroids.

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Toxicokinetics of 2,2′,4,4′- and 3,3′,4,4′-Tetrachlorobiphenyl in House Flies Following Topical Administration

SHAKIL A. SAGHIR, GARY D. KORITZ, AND LARRY G. HANSEN

Department of Veterinary Biosciences, University of Illinois, 2001 South Lincoln Avenue, Urbana, Illinois 61801

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The effect of chlorine position on the absorption, distribution, metabolism, and excretion of two tetrachlorobiphenyls was studied in the house fly. Adult female house flies (*Musca domestica* L.) were dosed topically with 0.5, 5, and 10 μg of 2,2′,4,4′-[^{14}C]- or 3,3′,4,4′-[^{14}C]tetrachlorobiphenyls (TCBs) in 1–2 μl of acetone. Total radioactivity in acetone rinses of intact flies, extracts of flies, extracts of excrements, and unextractable fly tissues and/or excrement was determined at 11 time points over a 96-hr period to provide data for the development of toxicokinetic models. Within 96 hr, 97–99% of the applied 2,2′,4,4′-TCB was absorbed from the surface following an initial rapid absorption of 93% within 12 hr. 3,3′,4,4′-TCB was absorbed very slowly, especially at the higher doses where only about 50% was absorbed within 96 hr. Both of the PCBs entered the fly body by simultaneous rapid (predominated for 2,2′,4,4′-TCB) and slow (predominated for the poorly soluble 3,3′,4,4′-TCB) absorption processes. A small fraction of the absorbed dose was irreversibly bound to fly tissues. A greater amount of 2,2′,4,4′-TCB was found in the fly body, but the amount of irreversibly bound radioactivity was higher for 3,3′,4,4′-TCB. Since almost all of the 2,2′,4,4′-TCB in the fly body was parent PCB, it is hypothesized that the greater burden but lower binding of 2,2′,4,4′-TCB in fly tissues is a result of a lack of reactive metabolites. Higher irreversible tissue binding of 3,3′,4,4′-TCB may reflect its greater metabolism. About 73% of the absorbed 3,3′,4,4′-TCB was excreted by 96 hr, whereas 76% of the absorbed 2,2′,4,4′-TCB was excreted at the lowest dose, decreasing to 56% at the highest dose. Selective absorption, excretion, tissue retention, and greater metabolism of the lower doses more representative of environmental exposure suggest that the large mobile biomass of insects may play a crucial role in the conversion of environmental PCBs to more polar metabolites as well as the dispersion of parent PCBs and their metabolites in food chains due to the dominant role of insects in many food webs. © 1994 Academic Press, Inc.

INTRODUCTION

Insects are the most numerous group in the animal kingdom, and the number of species exceeds all other animal groups combined. Ubiquitous and diverse, insects are dominant components of many food webs and occupy the critical middle links among the four or five trophic levels commonly present in many terrestrial and freshwater food chains (1). Because insects usually have a greater biomass in a given area than that of other mobile animals, they are particularly important in studies related to environmental pollution and the dispersion of pollutants.

Lipophilic chemicals such as polychlorinated biphenyls (PCBs) have been found in considerable concentrations in honeybees (2), the highest concentrations being detected in the wax of honeycombs (3). Crickets, salt marsh caterpillars, and June beetle larvae also accumulate residues of lipophilic chemicals (4–7). Among other terrestrial insects, wasps were found to have the highest PCB concentrations, perhaps acquired during consumption of PCB-containing prey insects (3).

Insects play an important role in the modification (4, 8) of persistent chemicals and dispersion of their metabolites. To accurately predict the impact of insects on the modification and movement of chemicals in ecosystems, detailed toxicokinetic models of uptake, metabolism, and excretion of prototype chemicals in individual insect species would be useful (9, 10). Toxicokinetic models should explain the distribution

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of chemicals in an ecosystem better than the common food chain approach (11).

Polychlorinated biphenyls are environmentally persistent and highly lipophilic compounds found at all levels of the food chain. PCBs accumulate and biomagnify in the food chain in the same manner as many chlorinated pesticides (8, 12, 13). Of 1.2×10^6 tons of PCBs produced, 400×10^6 kg is the estimated mobile environmental burden worldwide (12–15). PCBs may be transferred from especially soils and sediments (16) throughout the food web and eventually to man.

The properties of each of the 209 possible congeners are unique, depending on the degree and pattern of chlorination of the biphenyl rings (12, 13, 17–20). PCBs are metabolized to more polar products almost always by a cytochrome P_{450} monooxygenase (21). Each metabolite is also unique and capable of specific receptor interactions and/or covalent binding to tissues, cellular macromolecules, and nucleic acids (10, 12, 13, 22, 23). Therefore, the persistence, disposition, and toxicity of the individual congeners must be defined to assess the hazards associated with their presence in various environmental reservoirs (12, 13, 20).

House flies metabolize PCBs to more polar products (10, 24) and selectively retain certain congeners from PCB mixtures (25). This study was conducted to develop and compare a multicompartmental toxicokinetic model of the absorption, distribution, metabolism, and excretion of 2,2′,4,4′-[^{14}C]tetrachlorobiphenyl (2,2′,4,4′-TCB) and 3,3′,4,4′-[^{14}C]tetrachlorobiphenyl (3,3′,4,4′-TCB) in house flies following topical administration on the dorsal thorax. This information should be useful in the comparison of PCBs with different patterns of chlorination with respect to uptake, tissue accumulation, and biomagnification. It should also give better insight into the role of insects in the selective dispersion of PCB congeners from environmental reservoirs and clarify the results of earlier studies (10, 24–28). Furthermore, the information should add to the understanding of the toxicokinetics of other lipophilic chemicals in insects (29).

MATERIALS AND METHODS

Rearing of Flies

House flies of the NAIDM pesticide-susceptible strain (but with slight resistance to DDT) were reared at 30 ± 2°C from a colony maintained in the Department of Entomology, University of Illinois, Urbana–Champaign. Pupae were transferred to 0.3 × 0.3 × 0.3-m wire screen cages for emergence. Following emergence, the flies were fed *ad libitum* on sugar cubes, distilled water, and 10% powdered milk solution; milk was withdrawn after 2 days to reduce egg laying. Although this would alter the model to be less applicable to gravid females, the variable egg laying and PCB excretion in the egg would needlessly complicate modeling. At Day 5 following emergence (highest level of microsomal enzymes, (30)), the flies were anesthetized with carbon dioxide, and batches of three female house flies of uniform size were used in the experiment.

Chemicals

Uniformly radiolabelled 2,2′,4,4′-TCB, 2,2′,4,4′-[^{14}C]tetrachlorobiphenyl (MW 296, sp. act. 13.8 mCi/mmol), and 3,3′,4,4′-TCB, 3,3′,4,4′-[^{14}C]tetrachlorobiphenyl (MW 296, sp. act. 37.1 mCi/mmol) were purchased from Sigma Chemicals (St. Louis, MO). Both of the chemicals were greater than 99% pure.

Treatment of Flies

Doses were chosen to bracket a very narrow range within which 2,2′,4,4′-TCB exhibited a dramatic increase in toxicity to the housefly (28, 31) and 3,3′,4,4′-TCB could easily be administered without crystallizing at the tip of the applicator. A 0.5, 5, or 10 μg dose was applied topically to the dorsal thorax of each anesthetized fly in 1.0 or 2.0 μl acetone using a microsyringe fitted to a

manually operated vernier caliper. Following exposure, the flies were placed in groups of three in 20-ml scintillation vials lined with filter paper (Whatman No. 50) to trap excrement and reduce reabsorption of PCB by contact. Vials were covered with two layers of cheese cloth secured with rubber bands. Small filter paper discs were inserted between the cloth layers and wetted with sugar solution (20%) to supply food and water. The vials of each PCB experiment were kept in a single desiccator. A constant volume of air was drawn from the desiccator through two traps consisting of isooctane to collect volatile compounds followed by dilute NaOH to trap CO_2. The radioactivity collected in these traps was included in the mass balance accounting.

Analysis

At specified times, batches of three flies were anesthetized with CO_2, removed from the scintillation vial, rinsed with 5 ml acetone, sonicated (Heat Systems-Ultrasonics, Inc. W-225R sonicator), and extracted three times in 6 ml acetone:hexane (1:1). The filter paper lining of the vials was cut into small pieces and extracted three times to recover the PCB and its metabolites from excrement. All extracts were kept at −10°C until thin-layer chromatographic (TLC) analysis.

Twenty-five percent of each extract was counted for radioactivity (Packard Tri-Carb 1500M Scintillation counter), and the remainder was concentrated under a gentle stream of nitrogen gas and then spotted on 20 × 20 cm, 250-μm-thick Whatman's LK5F linear-K silica gel plates for thin-layer chromatography. The plates were developed with benzene:dioxane:acetic acid (64:34:1.5, v/v), as described elsewhere (10, 24, 32). Radioactive spots on the TLC plates were analyzed by exposing the plates for 3–5 days to an MD28 storage phosphor screen (Molecular Dynamics, Sunnyvale, CA) with detection by a phosphor imager (425S PhosphorImager, Molecular Dynamics). The masses of parent PCB and metabolites were quantitated by the relative densities of each spot at each time point using computer software [ImageQuant (Ver. 3.3), Molecular Dynamics, 1993]. Radioactivity in the filter paper lining that could not be extracted even after three rigorous extractions with acetone:hexane (1:1) was determined by counting the filter paper. Extracted fly tissues were digested (100 μl Protosol, New Research Products, Boston, MA), pH was adjusted by adding 100–200 μl of acetic acid to eliminate chemiluminescence, and the samples were then counted for unextractable radioactivity.

Kinetic Analysis

Averaged data of two separate experiments were subjected to toxicokinetic analysis using the SAAM-30 computer program (33). A multicompartment model with first-order absorption, binding, and elimination was fitted to the data expressed as the percentage of applied dose versus time with equal weighting. Several attempts were made to fit different compartmental models with saturable metabolism and differential weighting of the data. The best-fit model provided irreversible binding of some radioactivity in an unextractable compartment with elimination of the remaining radioactivity (parent PCB + metabolites) from the fly body and a fraction of the dose bypassing the fly body. Areas under the time–concentration curves (AUC) for parent PCBs and pooled metabolites were calculated by the trapezoidal method to the last data point. Half-lives ($t_{1/2}$) of the disposition of parent PCBs and pooled metabolites in flies were calculated from the terminal slopes (last 72 hr) of the semilog plots. Binding of parent PCBs and/or metabolites to fly tissues was calculated at 96 hr post-treatment as well as at infinite time based upon the parameters of the toxicokinetic models. Metabolite-to-parent AUC ratios for the fly extract, compartment 3, and cumulative percentage dose recovered from the excrement after 96 hr were calculated to compare the disposition of different com-

mon metabolites at different doses as well as in different compartments (fly extract and excrement).

RESULTS AND DISCUSSION

Toxicokinetic model. Figure 1 summarizes the movement of the topically applied PCBs in house flies. The applied PCBs (2,2',4,4'-[^{14}C]TCB and 3,3',4,4'-[^{14}C]TCB) move from the dorsal thorax into the fly body by rapid (K_{31}) and slow (K_{21}, K_{32}) absorption processes. A fraction of the absorbed dose binds irreversibly to the fly tissues with a rate constant of (K_{43}) and the rest is eliminated from the body as excrement (K_{53}). Some of the applied dose bypasses absorption and irreversibly binds to the tissues (K_{41}) or moves to the excrement (K_{51}). Several attempts were made to fit the model without the bypass compartments, but very poor fits were achieved in all cases. There was no loss of the applied dose from the system because of the low volatility of the PCBs used. A similar toxicokinetic model was proposed earlier for 2,2',5-[^{14}C]trichlorobiphenyl (2,2',5-TCB) in house flies; however, that model included loss of some of the applied dose from the system, because of the volatility of 2,2',5-TCB, and did not require the bypass processes (10). The rate constants of elimination of parent PCBs and their metabolites from flies could not be determined separately and, hence, they are presented as a single route of elimination (K_{53}).

Absorption. Decreases in radioactivity in the acetone rinse at increasing times after exposure reflect the rates of absorption of PCBs through the fly cuticle. 2,2',4,4'-TCB was absorbed very rapidly into the fly body; about 94% (94.4, 93.5, and 93.1% of 0.5, 5, and 10 μg/fly, respectively) of the applied dose disappeared from the fly surface within 12 hr (Figs. 2–4). The rate of absorption of the lower doses was very rapid at early time points; e.g., 51.9% of the applied dose of 0.5 μg/fly disappeared from the surface within 1 hr (Figs. 2–4). Rapid absorption of another *ortho*-substituted PCB (2,2',5-TCB) has been reported earlier (10).

The absorption of 3,3',4,4'-TCB was markedly slower than that of 2,2',4,4'-TCB, mostly because of very low solubilities of coplanar PCBs as evidenced by the crystallization of 3,3',4,4'-TCB at the dosing site. Only 53.5, 26.1, and 31.6% of the applied doses of 0.5, 5, and 10 μg/fly, respectively, were absorbed within 12 hr (Figs. 5–7). Extent of absorption at early

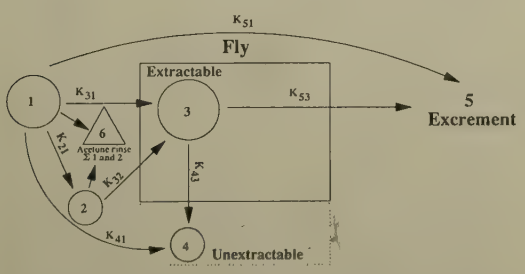

FIG. 1. *Toxicokinetic model of topically applied 2,2',4,4'-[^{14}C]TCB and 3,3',4,4'-[^{14}C]TCB in the house fly.*

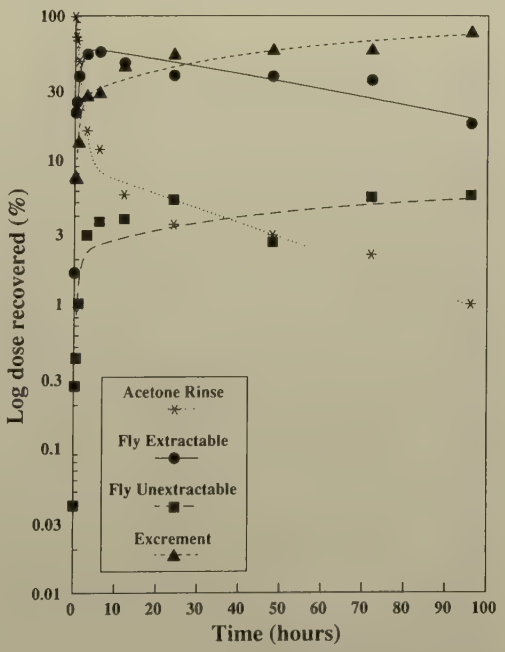

FIG. 2. *Time course of the absorption and disposition of 0.5 μg topically applied 2,2',4,4'-[^{14}C]TCB in house flies (n = 2 replicates of three flies per time point). Observed data points are presented and smooth lines represent fitted model values.*

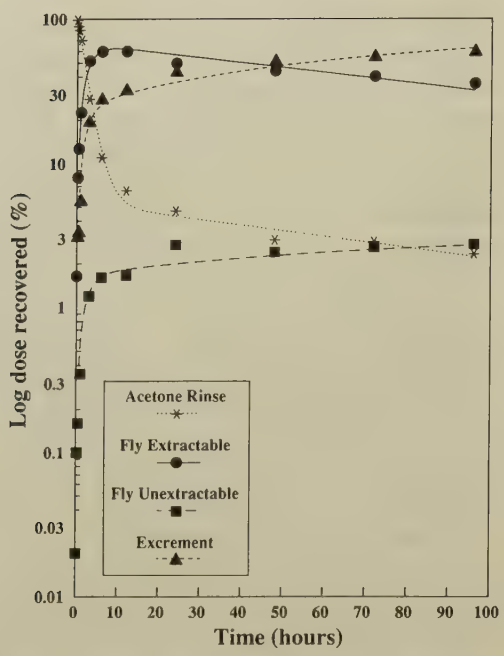

FIG. 3. *Time course of the absorption and disposition of 5 μg topically applied 2,2′,4,4′-[^{14}C]TCB in house flies (n = 2 replicates of three flies per time point). Observed data points are presented and smooth lines represent fitted model values.*

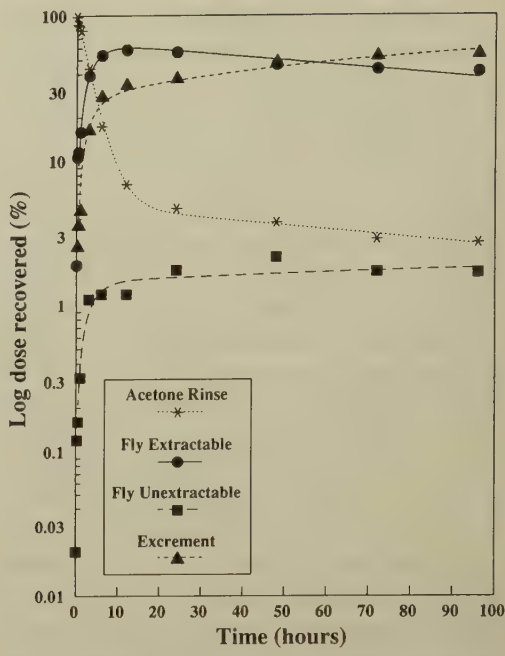

FIG. 4. *Time course of the absorption and disposition of 10 μg topically applied 2,2′,4,4′-[^{14}C]TCB in house flies (n = 2 replicates of three flies per time point). Observed data points are presented and smooth lines represent fitted model values.*

times was nearly the same at all doses. At 1 hr, 88.5, 95, and 91% of the doses of 0.5, 5, and 10 μg/fly, respectively, and 77, 91, and 81% of the doses at 3 hr remained to be absorbed (Figs. 5–7). About 50% of the dose remained unabsorbed even after 96 hr. Li and McKee (7) reported 25–30% lower penetration of 3,3′,4,4′-TCB within 24 hr than 2,2′,4,4′-TCB in house crickets treated with 0.1 μg of PCBs per cricket; the same results were found in the present study at the lowest (0.5 μg/fly) dose [29% lower penetration of 3,3′,4,4′-TCB than 2,2′,4,4′-TCB within 24 hr (Figs. 2 and 5)].

The absorption of both PCBs was biphasic as reported by Saghir *et al.* (10) and Storr-Hansen *et al.* (34). The early rapid absorption phase was accompanied by a slow absorption phase which may reflect diffusion of the applied PCBs into deeper cuticular layers and subsequent slower release from these layers (10). The half-lives of the fast absorption process for 2,2′,4,4′-TCB were 1.26, 2.39, and 3.44 hr at the three increasing doses and for the slow absorption process were 29.41, 83.2, and 109.48 hr at the three doses (Table 1). On the other hand, half-lives of the fast as well as the slow absorption processes for the poorly soluble 3,3′,4,4′-TCB were longer [8, 39, and 42 hr for the fast process and 23.1, 152.6, and 280.6 hr for the slow absorption process for the three increasing doses (Table 2)].

The rate of penetration of both PCBs into the deeper cuticular layers (K_{21}) was slower at higher doses (Tables 1 and 2). Also the rate constants of slow absorption from the deeper cuticular layers (K_{32}) and rapid absorption from the application site (K_{31}) were slower and their respective half-lives were longer at higher doses (Tables 1 and 2). Retardation of the rates of all the processes involved in absorption at higher doses (K_{31}, K_{21}, and K_{32}) might be due to the toxicity [especially for 2,2′,4,4′-TCB

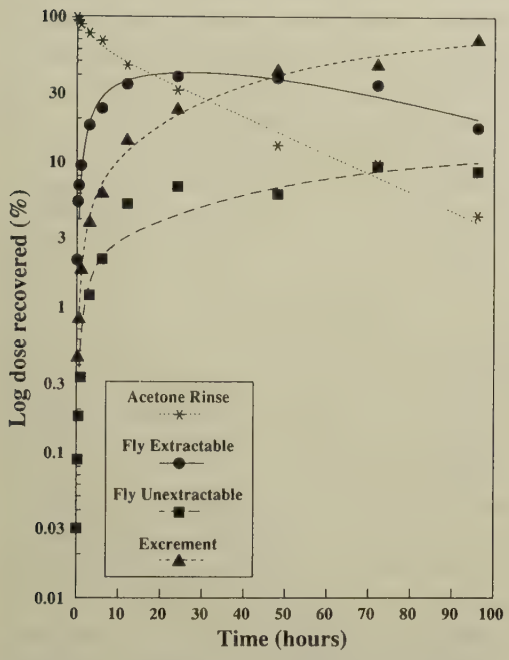

FIG. 5. *Time course of the absorption and disposition of 0.5 μg topically applied 3,3′,4,4′-[^{14}C]TCB in house flies (n = 2 replicates of three flies per time point). Observed data points are presented and smooth lines represent fitted model values.*

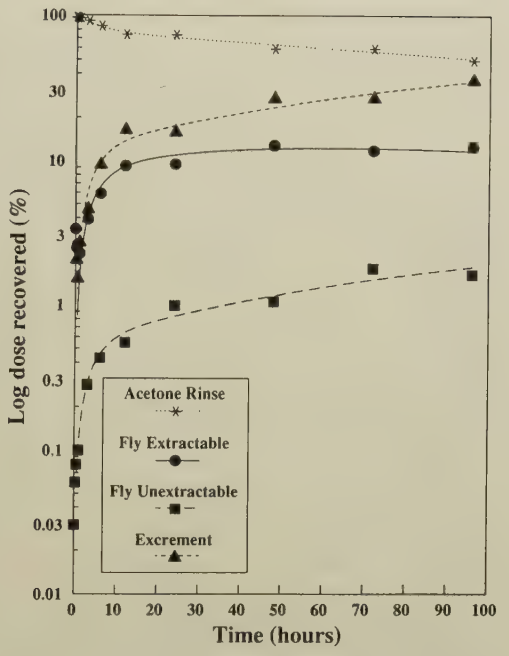

FIG. 6. *Time course of the absorption and disposition of 5 μg topically applied 3,3′,4,4′-[^{14}C]TCB in house flies (n = 2 replicates of three flies per time point). Observed data points are presented and smooth lines represent fitted model values.*

(31)], saturation of the system, or binding and/or partitioning of the applied PCBs within the fly integument (29). To model all of the processes involved in the absorption of the applied PCBs into the fly body, additional processes accounting for the slow absorption (K_{21}, K_{32}) have been introduced; thus, the rapid (K_{31}) as well as the slow (K_{21}, K_{32}) absorption processes were modeled simultaneously.

Most of the applied 2,2′,4,4′-TCB entered into the fly body via rapid (K_{31}) absorption and only a small fraction of the dose via slow (K_{21}) absorption (Table 3). In 3,3′,4,4′-TCB, the major route of absorption of the applied dose was the slow (K_{21}) process; only 30.5, 9, and 7.3% of the applied dose entered via the fast (K_{31}) absorption process at increasing doses (Table 3). The binding of 2,2′,5-TCB was similar at 10, 15, and 20 μg/fly, and no major differences in absorption were detected (10).

Bypass. Some of the applied dose bypassed the normal absorption processes or moved so rapidly through the fly body that it could not be modeled; therefore, two bypass processes (K_{41} and K_{51}) were introduced to the model (Fig. 1). Only 0.5–2.4% of the dose moved through K_{41} within 96 hr, and this bypass route contributed more at lower doses for both PCBs (Table 3). At every dose, 2,2′,4,4′-TCB had faster rates than 3,3′,4,4′-TCB (Tables 1 and 2). A total of about 28% of the applied 2,2′,4,4′-TCB disappeared from the fly surface within 96 hr via K_{51} with a rate of 0.25, 0.11, and 0.09/hr for increasing doses (Tables 1 and 3); 6, 14, and 29% of 3,3′,4,4′-TCB moved via K_{51} with rate constants of 0.02, 0.03, and 0.06/hr for the three doses, respectively (Tables 2 and 3).

Accumulation. Peak accumulation of total (extractable plus unextractable) radioactivity in the fly body of 2,2′,4,4′-TCB amounted to 60% of the applied dose and occurred at 6 hr for the 0.5 μg/fly dose and

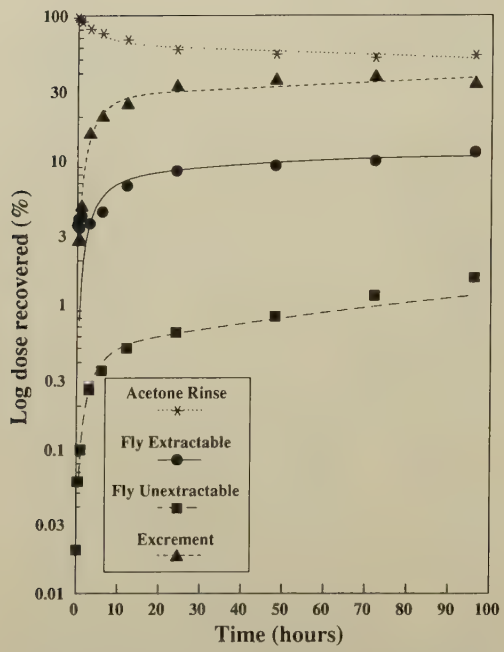

FIG. 7. *Time course of the absorption and disposition of 10 μg topically applied 3,3′,4,4′-[^{14}C]TCB in house flies (n = 2 replicates of three flies per time point). Observed data points are presented and smooth lines represent fitted model values.*

12 hr for 5 and 10 μg/fly doses (Figs. 2–4). Within 15 min after treatment, 8–22% (21.6, 8.1, and 10.8% at 0.5, 5, and 10 μg/fly, respectively) of the applied dose was found in the fly body (Figs. 2–4). The initial absorption of 2,2′,4,4′-TCB was faster than for 2,2′,5-TCB as reported earlier (10); however, 2,2′,4,4′-TCB remained in the fly body far longer than the highly metabolized 2,2′,5-TCB. The 3,3′,4,4′-TCB took longer to peak in the fly body: 24 hr for 0.5 μg/fly (45.6% of the applied dose) and had not yet peaked at 96 hr at 5 and 10 μg/fly (14.3 and 12.9% of the applied dose). Only 2–5% of the dose was found in the fly body 15 min after treatment (Figs. 5–7).

Ninety-six hours after treatment, 23.5, 38.7, and 43% of the applied 2,2′,4,4′-TCB and 26.2, 14.3, and 12.9% of the applied 3,3′,4,4′-TCB at 0.5, 5, and 10 μg/fly, respectively, remained in the fly body (Figs. 2–7). A greater retention of 2,2′,4,4′-TCB than 3,3′,4,4′-TCB was found in all the fly tissues (head, thoracic cuticle, thoracic muscles, abdominal muscles, and ovaries) with the exception of alimentary canal, at almost all of the time points. The lower concentration in the alimentary canal is due to lower excretion of 2,2′,4,4′-TCB (Saghir and Hansen, unpublished data). The 2,2′,4,4′-TCB is also retained more than 3,3′,4,4′-TCB in almost all tissues, with the exception of blood, in rats (23, 35, 36).

Unextractable compartment (4). Some 2,2′,4,4′-TCB as well as 3,3′,4,4′-TCB were firmly bound to the fly tissues and unextractable by the solvent (Fig. 1). Consequently, the model was fitted with irreversible binding of the radioactivity to the fly tissues (10). Covalent binding of PCBs to mammalian macromolecules has been reported (12, 22, 23, 35, 36). The highest amounts of 2,2′,4,4′-TCB (or its metabolites) in the unextractable compartment 4 were found at 96 hr for 0.5 and 5 μg/fly doses and 48 hr for 10 μg/fly (Figs. 2–4). Conversely, the peak amount of unextractable 3,3′,4,4′-TCB was found at 72 hr for 0.5 and 5 μg/fly and at 96 hr for 10 μg/fly (Figs. 5–7). The subsequent decline in unextractable radioactivity at some doses might have resulted from the normal continuous sloughing of the peritrophic membrane in the insect gut and radioactivity bound therein (37), as well as decreased input from compartment 3 (extractable fly). A decrease in the unextractable radioactivity from the fly tissues with increasing doses (Figs. 2–7) indicates the saturation of the metabolizing enzymes or a saturable binding process in the unextractable compartment of the fly. This is also evident from the decrease in the rate constant for the distribution of PCBs into the unextractable compartment (K_{43}) with increased amounts of PCBs in the extractable compartment (Tables 1 and 2). A similar saturable binding of 2,2′,5-TCB in the unextractable compartment has been reported earlier (10).

Although significantly greater amounts of 2,2′,4,4′-TCB were found in the fly body after 96 hr of treatment, the amount of un-

TABLE 1

Toxicokinetic Parameters for 2,2',4,4'-[^{14}C]TCB in House Flies Following Topical Administration of Three Different Doses

Parameters	Doses of 2,2',4,4'-[^{14}C]TCB		
	0.5 μg/fly	5 μg/fly	10 μg/fly
K_{31} (hr^{-1})	0.54880	0.28957	0.20161
K_{21} (hr^{-1})	0.08023	0.02261	0.01514
K_{32} (hr^{-1})	0.02356	0.00912	0.00669
K_{53} (hr^{-1})	0.01319	0.00833	0.00633
K_{51} (hr^{-1})	0.24910	0.11001	0.08700
K_{43} (hr^{-1})	0.00085	0.00022	0.00007
K_{41} (hr^{-1})	0.02055	0.00729	0.00454
$t_{1/2}$, Fast absorption (hr)	1.26	2.39	3.44
$t_{1/2}$, Slow absorption[a] (hr)	29.41	83.19	109.48
$t_{1/2}$, Disappearance[b] (hr)	49.35	83.15	112.30
	Compartment 3 (extractable fly)		
$t_{1/2}$, Elimination of total PCB[c]	49.36	81.05	108.28
AUC, Parent PCB (% dose hr)[d]	3463.83	4093.10	4388.63
$t_{1/2}$, Parent PCB[e] (hr)	71.44	151.97	168.20
AUC, Metabolites (% dose hr)[d]	65.92	218.89	159.74
$t_{1/2}$, Metabolites[e] (hr)	65.19	257.62	134.56

[a] $t_{1/2}$ (slow absorption) = $0.693/K_{32}$.
[b] Calculated from the simulated terminal disappearance phase, 700–1200 hr (0.693/slope).
[c] Calculated as $0.693/(K_{43} + K_{53})$.
[d] Calculated by the trapezoidal method to the last data point.
[e] Calculated from the terminal phase slope (last 72 hr).

TABLE 2

Toxicokinetic Parameters for 3,3',4,4'-[^{14}C]TCB in House Flies Following Topical Administration of Three Different Doses

Parameters	Doses of 3,3',4,4'-[^{14}C]TCB		
	0.5 μg/fly	5 μg/fly	10 μg/fly
K_{31} (hr^{-1})	0.08645	0.01779	0.01621
K_{21} (hr^{-1})	0.17233	0.15124	0.14049
K_{32} (hr^{-1})	0.02996	0.00454	0.00247
K_{53} (hr^{-1})	0.01921	0.02078	0.01017
K_{51} (hr^{-1})	0.01775	0.02673	0.06309
K_{43} (hr^{-1})	0.00246	0.00114	0.00071
K_{41} (hr^{-1})	0.00678	0.00122	0.00115
$t_{1/2}$, Fast absorption (hr)	8.02	38.95	42.75
$t_{1/2}$, Slow absorption[a] (hr)	23.13	152.64	280.57
$t_{1/2}$, Disappearance[b] (hr)	31.97	152.66	280.60
	Compartment 3 (extractable fly)		
$t_{1/2}$, Elimination of total PCB[c]	31.98	31.61	63.69
AUC, Parent PCB (% dose hr)[d]	2181.02	906.39	659.72
$t_{1/2}$, Parent PCB[e] (hr)	79.03	195.24	155.59
AUC, Metabolites (% dose hr)[d]	943.47	132.62	185.49

[a] $t_{1/2}$, (slow absorption) = $0.693/K_{32}$.
[b] Calculaed from the simulated terminal disappearance phase, 700–1200 hr (0.693/slope).
[c] Calculated as $0.693/(K_{43} + K_{53})$.
[d] Calculated by the trapezoidal method to the last data point.
[e] Calculated from the terminal phase slope (last 72 hr).

TABLE 3

Percentage Disappearance of the Applied 2,2′,4,4′-[^{14}C]TCB and 3,3′,4,4′-[^{14}C]TCB through Different Routes from the Surface of House Flies at Three Different Doses

Route	% Applied dose at three doses		
	0.5 μg/fly	5 μg/fly	10 μg/fly
	2,2′,4,4′-[^{14}C]Tetrachlorobiphenyl[a]		
K_{31}	61.07	67.42	65.40
K_{21}	8.93	5.26	4.91
K_{41}	2.29	1.70	1.47
K_{51}	27.72	25.61	28.22
	3,3′,4,4′-[^{14}C]Tetrachlorobiphenyl[b]		
K_{31}	30.51	9.03	7.34
K_{21}	60.83	76.78	63.59
K_{41}	2.39	0.62	0.52
K_{51}	6.27	13.57	28.56

[a] Within 96 hr, 98 ± 1% of the applied dose disappeared from the surface.

[b] Within 96 hr, 96, 50, 47% of the applied dose disappeared from the surface at 0.5, 5, and 10 μg/fly doses, respectively.

extractable radioactivity was lower than 3,3′,4,4′-TCB (Table 4). When the amount of unextractable radioactivity was extrapolated to infinite time based upon the toxicokinetic models (Fig. 1), the percentage unextractable dose for 3,3′,4,4′-TCB was higher at every dose and the tissue binding factor was almost twice that of 2,2′,4,4′-TCB (Table 4). A higher tissue burden and lower unextractable and/or covalent binding of 2,2′,4,4′-TCB compared to 3,3′,4,4′-TCB have also been reported in rats (23, 35, 36).

Extractable compartment (3). The total extractable 2,2′,4,4′-[^{14}C]TCB in compartment 3 peaked at 6 hr for 0.5 μg/fly and 12 hr for 5 and 10 μg/fly. Parent 2,2′,4,4′-TCB also peaked at these times (Table 5). The ratios of the molar concentrations of the total and parent 2,2′,4,4′-TCB among the doses were the same (Fig. 8; Table 5). The disposition rate constants of parent 2,2′,4,4′-TCB were slower at higher doses (0.0097, 0.0046, and 0.0041/hr for 0.5, 5, and 10 μg/fly doses, respectively), as calculated from the last 72 hr of data, and their corresponding half-lives were longer (Table 1). Elimination of the total (parent plus metabolites) PCB was also delayed with increasing doses, which is apparent from

TABLE 4

Unextractable and Binding Factors of 2,2′,4,4′-[^{14}C]TCB and 3,3′,4,4′-[^{14}C]TCB in House Flies

Dose (μg/fly)	96 hr		∞ hr[a]	
	Unextractable[b] (% dose)	BF[c] (% absorb/mg)	Unextractable[b] (% dose)	BF[c] (% absorb/mg)
	2,2′,4,4′-[^{14}C]Tetrachlorobiphenyl			
0.5	5.58[d]	0.41	6.52	0.48
5.0	2.74[d]	0.21	3.57	0.26
10	1.67[d]	0.13	2.24	0.16
	3,3′,4,4′-[^{14}C]Tetrachlorobiphenyl			
0.5	8.17[e]	0.67	12.76	0.94
5.0	1.62[e]	0.24	5.08	0.37
10	1.52[e]	0.24	5.15	0.37

[a] % Dose at infinite time, calculated as $[(K_{41}/(k_{21} + K_{31} + K_{41} + K_{51})) + ((K_{21} + K_{31})/(K_{21} + K_{31} + K_{41} + K_{51}))\ (K_{43}/(K_{43} + K_{53}))] \times 100$.

[b] % Applied dose unextractable from the fly body after grinding and rigorous extraction in acetone:hexane (1:1, v/v).

[c] Binding factor (% absorbed dose/mg wt) = radioactivity unextractable (% of the absorbed dose)/average fly weight (mg)

[d] Within 96 hr, 98 ± 1% of the applied dose was absorbed.

[e] Only 96, 50, and 47% of the applied dose actually absorbed within 96 hr at 0.5, 5, and 10 μg/fly doses, respectively.

TABLE 5

Peak Total Radioactivity, Parent Compound, and Metabolites in the Extracts of Flies Dosed Topically with 2,2′,4,4′-[^{14}C]TCB and 3,3′,4,4′-[^{14}C]TCB

Dose (μg/fly)	Time (hr)	Total (% dose (nmol))	Parent (% dose (nmol))	Metabolites (% dose (nmol))
		2,2′,4,4′-[^{14}C]Tetrachlorobiphenyl		
0.5	6	55.83 (0.95)	54.41 (0.93)	1.42 (0.02)
5.0	12	59.83 (10.21)	57.98 (9.87)	—
	24	—	—	2.61 (0.45)
10	12	58.40 (19.93)	56.79 (19.38)	—
	24	—	—	2.11 (0.72)
		3,3′,4,4′-[^{14}C]Tetrachlorobiphenyl		
0.5	24	38.88 (0.66)	26.01 (0.44)	12.87 (0.22)
5.0	48	12.85 (2.19)	11.32 (1.93)	—
	72	—	—	1.54 (0.26)
10	96	11.40 (3.89)	9.00 (3.07)	2.41 (0.82)

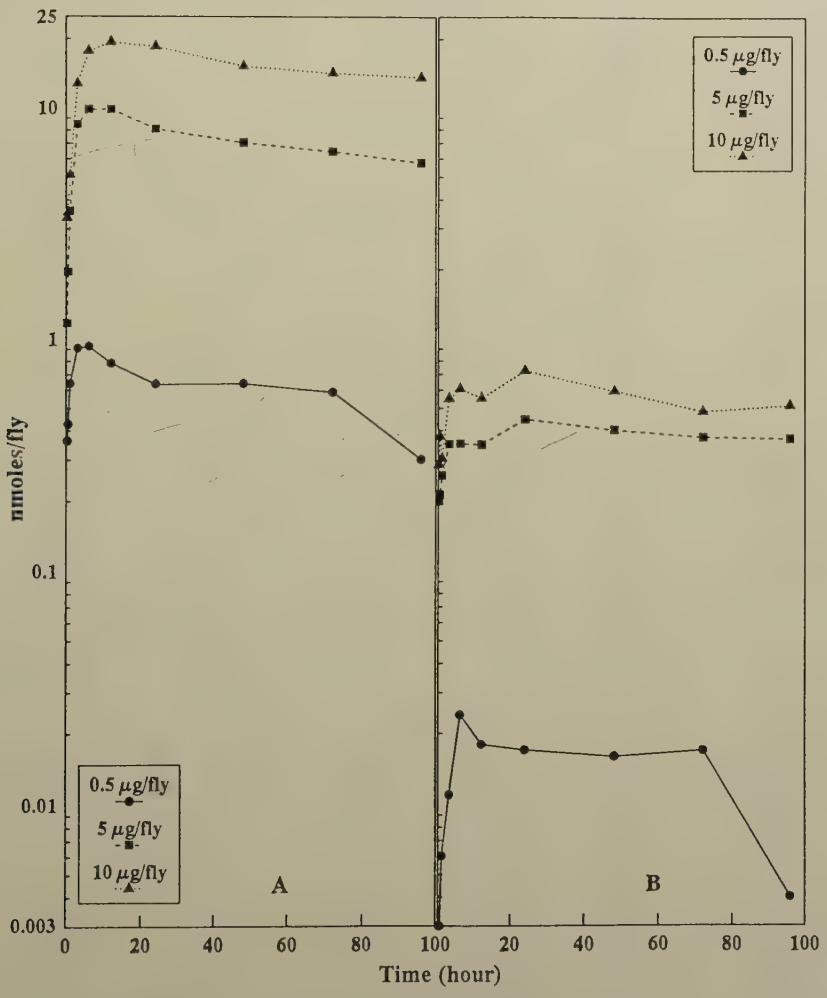

FIG. 8. *Molar disposition of parent (A) and total metabolites (B) found in extracts of flies dosed topically with three different concentrations of 2,2′,4,4′-[^{14}C]TCB.*

their longer half-lives (Table 1). Most of the applied 2,2′,4,4′-TCB could not be metabolized by house flies (Tables 6 and 7), and high amounts of parent PCB were found in the fly body. The peak amount of pooled metabolites of 2,2′,4,4′-TCB occurred at 6 hr for the 0.5 μg/fly dose and at 24 hr for the 5 and 10 μg/fly doses (Fig. 8; Table 5). The highest amounts of pooled metabolites in the fly body were only 1.2, 2.7, and 2.2% of the absorbed doses of 0.5, 5, and 10 μg/fly, respectively. Slowing of the disposition rate constant and increasing of the respective half-lives with an increase in dose could be due to rapid absorption followed by very little metabolism as a result of saturation of biotransformation enzymes (10).

The highest amounts of parent 3,3′,4,4′-TCB in the extractable compartment of the fly were found at 24 hr at 0.5 μg/fly; the higher doses appeared to plateau rather than peak with the highest amounts found at 48 hr for the 5 μg/fly and 96 hr for 10 μg/fly doses (Fig. 9; Table 5). The rate con-

TABLE 6

Recovery of Radioactivity in Fly Extract as a Percentage of Administered 2,2′,4,4′-[^{14}C]TCB Including Metabolites

Time (hr)	Total % dose	% Metabolites at different doses with R_f values					
		Polar 0.00	0.12	0.15	0.32	0.39	Parent 0.84
				0.5 μg/fly			
0.25	21.34	0.07	0.04	0.00	0.03	0.01	21.19
0.5	25.18	0.09	0.04	0.01	0.03	0.01	24.99
1	37.86	0.05	0.07	0.02	0.03	0.19	37.50
3	53.85	0.04	0.26	0.05	0.13	0.14	53.23
6	55.83	0.08	0.59	0.07	0.08	0.60	54.41
12	46.86	0.05	0.51	0.12	0.05	0.08	46.06
24	38.30	0.09	0.50	0.13	0.03	0.25	37.31
48	37.71	0.02	0.15	0.01	0.00	0.03	37.50
72	35.45	0.07	0.71	0.23	0.00	0.00	34.44
96	17.90	0.07	0.18	0.00	0.00	0.00	17.65
				5 μg/fly			
0.25	8.01	0.27	0.28	—[a]	0.30	0.31	6.84
0.5	12.73	0.28	0.30	—	0.33	0.34	11.48
1	22.56	0.32	0.36	—	0.41	0.41	21.05
3	51.59	0.40	0.55	—	0.58	0.51	49.55
6	59.72	0.38	0.66	—	0.57	0.46	57.66
12	59.83	0.41	0.65	—	0.53	0.45	57.79
24	49.66	0.48	0.75	—	0.55	0.57	47.30
48	43.93	0.56	0.83	—	0.59	0.64	41.32
72	40.05	0.47	0.72	—	0.48	0.52	37.86
96	35.97	0.48	0.72	—	0.46	0.49	33.81
				10 μg/fly			
0.25	10.63	0.20	0.21	—	0.21	0.22	9.80
0.5	11.48	0.26	0.27	—	0.28	0.30	10.37
1	15.89	0.20	0.22	—	0.23	0.23	15.00
3	38.93	0.34	0.42	—	0.42	0.42	37.32
6	53.68	0.42	0.49	—	0.46	0.40	51.90
12	58.40	0.37	0.51	—	0.40	0.32	56.79
24	56.41	0.47	0.78	—	0.46	0.41	54.30
48	46.30	0.37	0.58	—	0.38	0.40	44.57
72	43.05	0.30	0.49	—	0.30	0.32	41.64
96	41.44	0.31	0.65	—	0.27	0.27	39.94

[a] Not found.

TABLE 7
Recovery of Radioactivity in Fly Excrement as a Percentage of Administered 2,2′,4,4′-[^{14}C]TCB Including Metabolites

Time (hr)	Total % dose	% Metabolites at different doses with R_f values								
		Polar 0.00	0.12	0.20	0.32	0.39	0.50	0.69	Parent 0.84	Unext[a]
					0.5 μg/fly					
0.25	7.47	0.01	0.07	0.00	0.04	0.14	0.10	0.00	6.95	0.20
0.5	7.23	0.06	0.07	0.06	0.05	0.23	0.17	0.02	6.34	0.23
1	13.02	0.21	0.07	0.07	0.10	0.19	0.20	0.23	11.81	0.15
3	27.29	1.13	0.94	0.64	0.67	0.72	0.68	0.39	20.76	1.36
6	28.71	2.10	1.26	0.49	0.63	2.54	0.73	0.33	18.85	1.78
12	43.67	7.29	3.07	0.64	0.90	2.45	1.11	0.92	21.65	5.63
24	53.03	2.90	2.32	0.72	0.95	3.96	1.29	0.65	27.44	12.80
48	56.72	7.75	3.56	1.76	1.49	5.60	1.78	1.50	15.64	17.65
72	56.97	7.78	3.40	1.75	1.70	4.79	2.03	1.10	15.49	18.93
96	75.54	4.30	3.56	2.16	2.16	9.77	4.64	2.71	21.51	24.72
					5 μg/fly					
0.25	3.06	0.04	0.00	—[b]	0.00	0.01	0.00	—	2.91	0.09
0.5	3.31	0.03	0.00	—	0.00	0.01	0.00	—	3.14	0.12
1	5.48	0.09	0.02	—	0.00	0.08	0.00	—	5.05	0.25
3	19.31	0.07	0.16	—	0.03	0.10	0.00	—	18.16	0.78
6	27.62	1.54	0.64	—	0.07	0.75	0.00	—	22.78	1.84
12	32.02	1.35	0.74	—	0.16	0.56	0.02	—	25.73	3.46
24	42.94	2.74	1.64	—	0.39	1.75	0.16	—	27.17	9.10
48	50.74	5.44	2.00	—	0.33	2.83	0.27	—	24.05	15.83
72	54.50	4.51	3.21	—	0.53	3.80	0.53	—	21.15	20.77
96	58.97	5.82	4.14	—	0.58	4.49	0.59	—	23.88	19.47
					10 μg/fly					
0.25	2.53	0.00	0.00	—	0.00	0.00	—	—	2.49	0.04
0.5	3.56	0.00	0.00	—	0.00	0.00	—	—	3.47	0.09
1	4.54	0.06	0.01	—	0.04	0.00	—	—	4.30	0.12
3	16.31	0.09	0.04	—	0.03	0.00	—	—	15.81	0.34
6	27.76	0.27	0.27	—	0.20	0.00	—	—	25.83	1.19
12	33.50	0.53	0.67	—	0.44	0.05	—	—	29.11	2.69
24	37.13	1.89	1.63	—	0.86	0.11	—	—	27.56	5.09
48	47.80	4.68	3.05	—	1.37	0.33	—	—	26.80	11.57
72	52.39	4.98	5.47	—	2.92	0.50	—	—	25.01	13.51
96	54.20	2.61	3.64	—	2.93	0.41	—	—	30.25	14.36

[a] Unextractable from the filter paper lining.
[b] Not found.

stants of disposition of parent 3,3′,4,4′-TCB from the fly body were slower (0.0088, 0.0035, and 0.0045/hr of 0.5, 5, and 10 μg/fly, respectively) and the half-lives were longer with increasing doses, as calculated from the last 72 hr of the terminal phase (Table 2). As with extractable parent TCB, pooled metabolites of 3,3′,4,4′-TCB peaked at 24 hr for the 0.5 μg/fly, 72 hr for 5 μg/fly, and 96 hr for 10 μg/fly doses (Fig. 9; Table 5). About 18% of the absorbed 0.5 μg/fly PCB was found in the fly body in the form of metabolites at 24 hr, whereas only 3.8 and 5.2% of 5 and 10 μg/fly doses were found as metabolites in the fly at 72 and 96 hr, respectively (Table 8). The half-lives of the elimination of the total radioactivity from the body of house flies were almost identical (32 hr) at 0.5 and 5 μg/fly and twice that (64 hr) at the highest, 10 μg/fly,

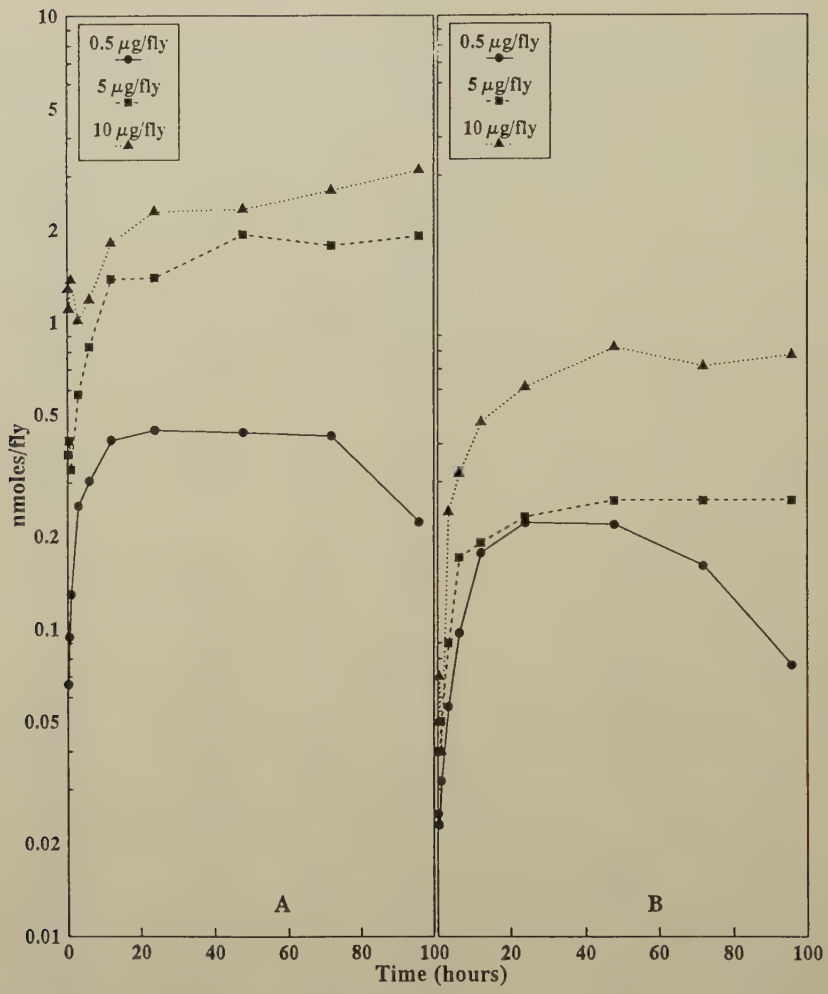

FIG. 9. *Molar disposition of parent (A) and total metabolites (B) found in extracts of flies dosed topically with three different concentrations of 3,3',4,4'-[^{14}C]TCB.*

dose (Table 2). The 3,3',4,4'-TCB was metabolized by the fly very efficiently (Tables 8 and 9), and lower metabolism at higher doses seems to be due to the very slow absorption of the dose from the application site and/or saturation of the biotransformation enzymes. Either or both could account for the longer $t_{1/2}$ of elimination at 10 μg/fly and the slowing of the disposition rate constant of the parent PCB with an increase in the dose (Table 2).

Metabolite elimination. The area under the curve (AUC) for parent as well as the pooled metabolites of 2,2',4,4'-TCB increased as the dose increased, but the increase in parent PCB relative to pooled metabolites (Table 1) indicated very little metabolism of 2,2',4,4'-TCB by the house fly and saturation of the metabolizing enzymes. Also, metabolite elimination was more impaired at lower doses than metabolite formation which caused an increased $t_{1/2}$ of the pooled metabolites at the 5 μg/fly dose (Table 1). A relationship between increasing AUC of parent PCB with increasing doses and saturation of the biotransformation enzymes has been reported earlier (10).

The AUC for parent and pooled metabolites of 3,3',4,4'-TCB decreased with increasing doses (Table 2). This demon-

TABLE 8

Recovery of Radioactivity in Fly Extract as a Percentage of Administered 3,3′,4,4′-[^{14}C]TCB Including Metabolites

Time (hr)	Total % dose	% Metabolites at different doses with R_f values							
		Polar 0.00	0.12	0.17	0.22	0.43	0.50	0.70	Parent 0.89
				0.5 μg/fly					
0.25	5.31	0.14	0.01	0.18	0.42	0.22	0.16	0.34	3.84
0.5	6.85	0.16	0.13	0.07	0.33	0.19	0.20	0.28	5.49
1	9.42	0.26	0.10	0.11	0.15	0.12	0.32	0.83	7.54
3	17.99	0.38	0.20	0.06	0.42	0.62	1.09	0.50	14.71
6	23.42	0.12	0.36	0.25	0.45	1.78	1.92	0.77	17.76
12	34.45	0.63	0.53	0.89	0.00	4.55	3.55	0.16	24.14
24	38.88	0.60	0.63	1.24	0.27	4.17	4.77	1.18	26.01
48	38.26	0.89	0.86	1.12	0.06	4.57	3.49	1.72	25.55
72	34.19	0.29	0.52	1.13	0.39	3.22	2.10	1.67	24.87
96	17.48	0.33	0.40	0.54	0.46	1.47	1.01	0.25	13.02
				5 μg/fly					
0.25	2.45	0.01	0.04	0.02	0.01	0.02	0.00	0.16	2.19
0.5	2.59	0.01	0.02	0.01	0.01	0.02	0.00	0.15	2.37
1	2.25	0.00	0.03	0.01	0.01	0.02	0.00	0.24	1.93
3	3.91	0.00	0.01	0.01	0.01	0.09	0.03	0.33	3.41
6	5.89	0.02	0.05	0.03	0.01	0.25	0.16	0.50	4.87
12	9.22	0.01	0.07	0.06	0.02	0.30	0.18	0.50	8.08
24	9.47	0.00	0.12	0.14	0.03	0.44	0.26	0.36	8.12
48	12.85	0.00	0.13	0.13	0.02	0.54	0.38	0.32	11.32
72	11.88	0.00	0.13	0.20	0.04	0.56	0.35	0.25	10.34
96	12.63	0.00	0.07	0.15	0.08	0.59	0.37	0.24	11.11
				10 μg/fly					
0.25	3.88	0.00	0.01	0.02	—[a]	0.06	0.05	—	3.74
0.5	3.41	0.01	0.05	0.08	—	0.02	0.03	—	3.21
1	4.14	0.01	0.01	0.01	—	0.07	0.01	—	4.01
3	3.64	0.06	0.07	0.01	—	0.43	0.11	—	2.95
6	4.39	0.08	0.08	0.02	—	0.53	0.22	—	3.45
12	6.68	0.11	0.19	0.04	—	0.82	0.21	—	5.30
24	8.48	0.07	0.24	0.05	—	1.04	0.37	—	6.70
48	9.23	0.09	0.27	0.21	—	1.15	0.68	—	6.82
72	9.92	0.07	0.26	0.24	—	1.01	0.51	—	7.84
96	11.40	0.06	0.35	0.29	—	0.96	0.60	—	9.13

[a] Not found.

strated slow absorption of the applied PCB at higher doses and not the inability of flies to metabolize 3,3′,4,4′-TCB [the AUC of parent PCB was only two- to sevenfold higher than that of the pooled metabolites (Table 2)]. The half-lives of the pooled metabolites for 3,3′,4,4′-TCB could not be determined because the curves were very flat, especially at 5 μg/fly (Fig. 9).

Total elimination. Most of the applied 2,2′,4,4′-TCB was rapidly eliminated from the body as parent compound. About 76% of the 0.5 μg/fly dose was found in the excrement after 96 hr (Fig. 2). Elimination decreased at higher doses; only about 59 and 54% of the 5 and 10 μg/fly doses, respectively, were excreted by 96 hr (Figs. 3 and 4). Even fewer metabolites of 2,2′,4,4′-TCB were found in the excrement with increasing doses (Table 7). The rate constants of elimination of total 2,2′,4,4′-TCB (parent PCB + metabolites) from the fly (K_{53}) also

TABLE 9

Recovery of Radioactivity in Fly Excrement as a Percentage of Administered 3,3′,4,4′-[^{14}C]TCB Including Metabolites

Time (hr)	Total % dose	% Metabolites at different times and doses with R_f values									
		Polar 0.00	0.12	0.17	0.22	0.34	0.43	0.50	0.70	Parent 0.89	Unext[a]
						0.5 μg/fly					
0.25	0.45	0.08	0.05	0.12	0.10	0.00	0.00	0.00	0.03	0.00	0.06
0.5	0.82	0.09	0.03	0.04	0.02	0.00	0.00	0.00	0.08	0.51	0.05
1	1.79	0.07	0.02	0.03	0.01	0.00	0.00	0.00	0.00	1.57	0.08
3	3.76	0.23	0.06	0.10	0.01	0.00	0.08	0.00	0.06	2.98	0.23
6	5.98	0.20	0.14	0.04	0.10	0.06	0.26	0.00	0.15	4.48	0.55
12	13.94	1.50	0.40	0.42	0.11	0.71	0.70	0.41	0.29	5.53	3.86
24	23.06	2.84	0.71	0.71	0.38	1.09	1.20	0.51	0.52	6.80	8.28
48	42.64	6.29	1.11	1.07	0.59	2.08	2.44	1.18	1.43	10.09	16.36
72	46.65	7.12	1.27	1.59	1.15	3.43	4.26	1.56	1.76	14.71	9.81
96	69.53	8.38	1.53	1.98	1.52	5.98	7.29	2.11	2.25	17.87	20.62
						5 μg/fly					
0.25	2.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	—[b]	1.94	0.10
0.5	1.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	—	1.40	0.11
1	2.69	0.00	0.00	0.00	0.00	0.00	0.00	0.00	—	2.54	0.15
3	4.58	0.00	0.00	0.00	0.00	0.00	0.00	0.00	—	4.29	0.30
6	9.36	0.55	0.19	0.00	0.00	0.00	0.00	0.00	—	8.01	0.60
12	16.37	2.22	0.45	0.08	0.16	1.07	0.63	0.00	—	10.63	1.12
24	15.83	0.84	0.29	0.18	0.22	1.11	0.82	0.18	—	10.38	1.83
48	26.98	1.56	0.42	0.64	0.53	2.54	2.88	0.72	—	13.31	4.37
72	27.04	2.35	0.31	0.82	0.58	2.52	3.02	0.89	—	9.05	7.50
96	36.15	4.35	0.37	0.49	0.81	3.02	4.10	0.76	—	13.51	8.74
						10 μg/fly					
0.25	2.71	0.00	0.00	0.00	0.00	0.00	0.00	0.00	—	2.58	0.13
0.5	2.82	0.00	0.00	0.00	0.00	0.00	0.00	0.00	—	2.70	0.12
1	4.75	0.09	0.00	0.00	0.00	0.00	0.05	0.00	—	4.45	0.17
3	15.15	0.40	0.11	0.02	0.07	0.21	0.13	0.13	—	13.58	0.49
6	19.96	0.37	0.21	0.04	0.07	0.40	0.41	0.37	—	17.47	0.62
12	24.42	0.33	0.10	0.08	0.15	0.56	0.53	0.29	—	21.56	0.82
24	32.38	2.03	0.58	0.48	0.58	2.05	1.91	0.88	—	22.07	1.80
48	35.79	2.48	0.81	0.75	0.79	3.24	3.53	1.23	—	19.76	3.22
72	37.64	2.15	0.92	0.90	0.96	3.22	3.74	1.80	—	19.45	4.49
96	33.78	2.17	0.52	0.64	0.78	2.66	3.23	1.69	—	17.76	4.34

[a] Unextractable from the filter paper lining.

[b] Not found.

decreased and the half-lives increased with increased doses (Table 1). Elimination at the early times (up to 12 hr) was almost the same as for 2,2′,5-TCB (10) which also slowed down by about 35% at later times (24 and 48 hr).

Only 96, 50, and 47% of the applied 3,3′,4,4′-TCB was actually absorbed into the fly body within 96 hr at 0.5, 5, and 10 μg/fly doses, respectively; nevertheless, about 73% of the absorbed doses was excreted from the body within 96 hr (Figs. 5–7). The rate constants of elimination of total 3,3′,4,4′-TCB from the fly (K_{53}) were about the same for 0.5 and 5 μg/fly and slightly decreased at the 10 μg/fly dose (Table 2). Half-lives of disappearance of the total 3,3′,4,4′-TCB increased with increasing doses (Table 2). Half-lives calculated from 700–1200 hr of the simulated terminal

disappearance phases and calculated from the elimination rate constants (K_{43} + K_{53}) were identical for the 0.5 μg/fly dose, whereas the former was 4.8- and 4.4-fold higher at 5 and 10 μg/fly doses, respectively (Table 2). At higher doses, half-lives of disappearance are the same as the half-lives of slow absorption, which indicates a flip-flop model wherein disappearance is controlled by the rate of slow absorption from the deeper cuticular layers (K_{32}) and not by the fast absorption rate constant as in the disappearance of 2,2′,4,4′-TCB [half-lives calculated from 700–1200 hr of the simulated terminal disappearance phases and elimination rate constants (K_{43} + K_{53}) are almost the same at every dose level (Table 1)].

Biotransformation. PCBs are metabolized mainly to mono- and dihydroxy biphenyls (21, 38) depending on the amount and activity of microsomal enzymes as well as the number and position of chlorine atoms on the biphenyl rings. Tables 6 and 8 show the disposition of 2,2′,4,4′-TCB and 3,3′,4,4′-TCB as different metabolites in the fly extract (compartment 3) and their respective R_f values. Only 0.15–2.6% of the applied 2,2′,4,4′-TCB was found as metabolites in the fly body (Table 6); lower amounts of metabolism are also apparent from the representative autoradiogram of the TLC separation of fly extracts (Fig. 10). The number of 2,2′,4,4′-TCB metabolites was fewer than for 3,3′,4,4′-TCB and became even fewer at higher doses (Tables 6–9). The highest amount of 2,2′,4,4′-TCB metabolites in the fly extract was found at 5 μg/fly and the least at 0.5 μg/fly (Table 6). The lower amount of extractable metabolites at 0.5 μg/fly might be due to covalent binding of the metabolites to the fly tissues, since the highest amounts of unextractable radioactivity were found at this dose, decreasing with increasing doses (Figs. 2–4). The binding sites may have been saturated at higher doses so that higher amounts of the metabolites became extractable. Almost all of the 2,2′,4,4′-TCB metabolites appeared in the fly extracts within 15 min

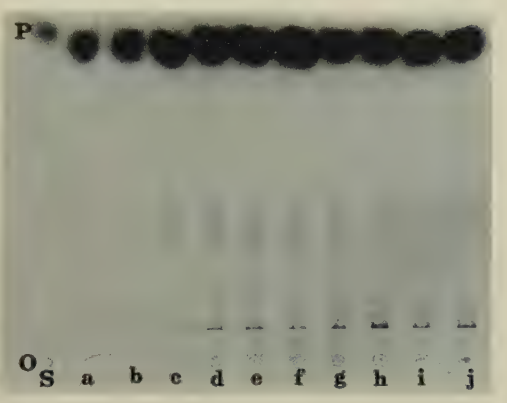

FIG. 10. *Representative autoradiogram of thin-layer chromatographic separation of extracts of flies at various times following topical dosing with 0.5 μg 2,2′,4,4′-[^{14}C]TCB. The surfaces of the flies were washed with acetone prior to extraction. Lane S is standard 2,2′,4,4′-[^{14}C]TCB. Lanes a–j are extracts at 0.25, 0.5, 1, 3, 6, 12, 24, 48, 72, and 96 hr, respectively. Concentrations of extracts were spotted at O (origin); P, parent PCB.*

following treatment in all three doses with the exception of the metabolite at R_f 0.15, which appeared 30 min after treatment at 0.5 μg/fly and could not be found at higher doses (Table 6). Earlier (within 5 min) appearance of 2,2′,5-TCB metabolites have been reported elsewhere (24). Much more rapid metabolism of the lower chlorinated PCB (2,2′,5-Tri-CB) compared to 2,2′,5,5′-TetraCB and 2,2′,4′,5,5′-PentaCB has been reported earlier (8, 32).

Greater numbers and amounts of 3,3′,4,4′-TCB metabolites were present in fly extracts (Table 8). Most of the radioactivity associated with 3,3′,4,4′-[^{14}C]TCB metabolites in the fly extract was intermediate in polarity with R_f values of 0.43 and 0.50 (Table 8 and Fig. 11). Metabolism of 3,3′,4,4′-TCB also decreased at higher doses, and two metabolites (R_f 0.22 and 0.70) could not be detected at 10 μg/fly (Table 8). Most of the metabolites appeared within 15 min following treatment and peaked at 48 hr at all three doses. The appearance and disappearance of different metabolites in the fly body may be observed in Fig. 11, an autoradiogram of a TLC plate.

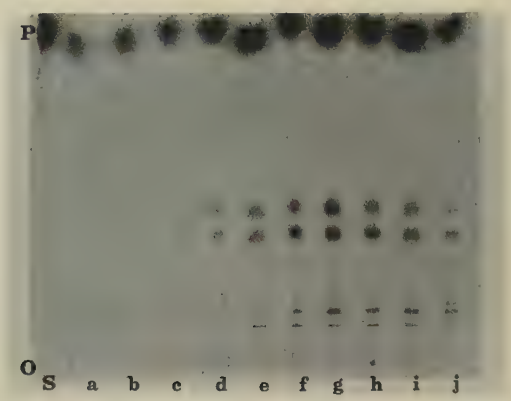

FIG. 11. *Representative autoradiogram of thin-layer chromatographic separation of extracts of flies at various times following topical dosing with 0.5 μg 3,3′,4,4′-[^{14}C]TCB. The surfaces of the flies were washed with acetone prior to extraction. Lane S is standard 3,3′,4,4′-[^{14}C]TCB. Lanes a–j are extracts at 0.25, 0.5, 1, 3, 6, 12, 24, 48, 72, and 96 hr, respectively. Concentrations of extracts were spotted at O (origin); P, parent PCB.*

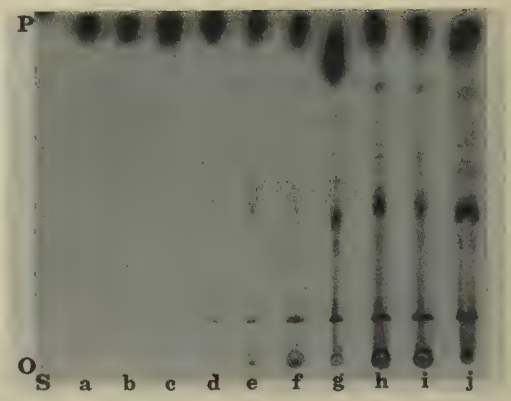

FIG. 12. *Representative autoradiogram of thin-layer chromatographic separation of extracts of fly excrements at various times following topical dosing with 0.5 μg 2,2′,4,4′-[^{14}C]TCB. Lane S is standard 2,2′,4,4′-[^{14}C]TCB. Lanes a–j are extracts at 0.25, 0.5, 1, 3, 6, 12, 24, 48, 72, and 96 hr, respectively. Concentrations of extracts were spotted at O (origin); P, parent PCB.*

One fairly polar metabolite of 2,2′,4,4′-TCB (R_f = 0.15) found in fly extracts (Table 6) of 0.5 μg/fly dose was not found in fly excrement (Table 7). Three 2,2′,4,4′-TCB metabolites (R_f = 0.20, 0.50, and 0.69) appeared in the excrement from low-dose flies, but were not detected in the fly body extracts at any dose. Metabolites at R_f 0.20 and 0.69 were only found at 0.5 μg/fly dose; metabolite at R_f 0.50 was not found at the highest (10 μg/fly) dose (Table 7). Most of the metabolites appeared in the excrement immediately (within 15 min) after dosing at the lowest (0.5 μg/fly) dose, but were delayed at higher doses (Table 7; Fig. 12).

A total of eight different metabolites of 3,3′,4,4′-TCB were found in the fly excrement (Table 9). One metabolite of intermediate polarity (R_f = 0.34) found in the fly excrement (Table 9) was not detected in the fly extract (Table 8). All of the metabolites in the excrement were common to all three doses with the exception of one metabolite found at R_f 0.70, which appeared only at the lowest (0.5 μg/fly) dose (Table 9). Three of the metabolites (R_f = 0.34–0.50) at the 0.5 μg/fly dose appeared 3 hr after dosing, whereas the others appeared immediately (Table 9). The appearance of all of the metabolites of 3,3′,4,4′-TCB was delayed by 3 hr at higher doses (Table 9), but is very apparent in the representative autoradiogram of the TLC plate (Fig. 13). The delay was greater than for 2,2′,4,4′-TCB and similar to 2,2′,5-TCB (10). Metabolites at the origin (R_f = 0.00) and at R_f 0.34 and 0.43 dominated (Table 9; Fig. 13). Metabolites

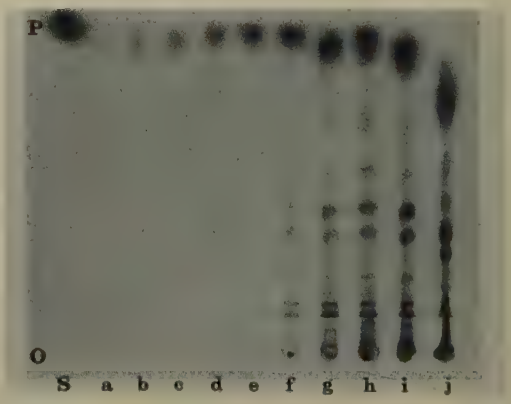

FIG. 13. *Representative autoradiogram of thin-layer chromatographic separation of extracts of fly excrements at various times following topical dosing with 0.5 μg 3,3′,4,4′-[^{14}C]TCB. Lane S is standard 3,3′,4,4′-[^{14}C]TCB. Lanes a–j are extracts at 0.25, 0.5, 1, 3, 6, 12, 24, 48, 72, and 96 hr, respectively. Concentrations of extracts were spotted at O (origin); P, parent PCB.*

less polar than the parent PCBs, as reported for the highly metabolizable 2,2′,5-tri-CB (8, 10), were not detected in the fly extracts or excrements from these tetra-CB-treated flies.

Unextractable excrement. Some of the excreted radioactivity from tetra-CB-treated flies could not be extracted from the filter paper lining of the scintillation vials even after rigorous acetone:hexane extraction and was reported as unextractable (Tables 7 and 9). A higher fraction (33%) of the excreted (or 24.7% of the applied) 2,2′,4,4′-TCB was trapped in the filter paper lining at lower doses (Table 7). The same pattern was found in the 3,3′,4,4′-TCB excrement; 30, 24, and 13% of the excreted doses were bound to the filter paper lining at 0.5, 5, and 10 μg/fly doses, respectively (Table 9). At the lowest dose the amount of unextractable radioactivity exceeded the parent radioactivity by about 13% for both tetra-CBs (Tables 7 and 9). Trapping of about 50% of the excreted 2,2′,5-tri-CB radioactivity on the filter paper lining and its extractability into more polar solvents [chloroform:methanol (2:1) and deionized water (pH 7.8)] has been reported earlier (10, 24). This suggests that nearly all of the trapped radioactivity consisted of polar metabolites. Polar solvents were not used in these tetra-CB studies, but the higher proportion of radioactivity not extractable from the filter paper lining into nonpolar solvents at the lower doses also suggests a greater extent of metabolism of these tetra-CBs at lower doses (Tables 7 and 9).

Tables 10 and 11 show the metabolite-to-parent (M/P) ratios in fly extracts and excrement from 2,2′,4,4′-TCB- and 3,3′,4,4′-TCB-treated flies. The M/P ratios of metabolites commonly found in the excrement decreased at higher doses (Tables 10 and 11). Predominantly parent TetraCBs were excreted at all three doses (Tables 7 and 9); the ratio of none of the metabolites was higher than 0.39 for 2,2′,4,4′-TCB (Table 10). This ratio reached 0.47 for polar 3,3′,4,4′-TCB metabolites and about 1.2 for

TABLE 10

Relative Amounts (Metabolite/Parent (M/P)) of Individual Metabolites Excreted by House Flies during the 96-hr Interval Following Topical Application of 2,2′,4,4′-[^{14}C]TCB and Metabolite/Parent Area under the Curve (AUC) for Fly Extracts during this Time Interval

R_f	Doses of 2,2′,4,4′-[^{14}C]TCB		
	0.5 μg/fly	5 μg/fly	10 μg/fly
M/P compartment 5 (excrement) at 96 hr			
0.00	0.33	0.23	0.14
0.12	0.19	0.16	0.16
0.20	0.11	—[a]	—
0.32	0.10	0.02	0.11
0.39	0.39	0.18	0.02
0.50	0.18	0.02	—
0.69	0.10	—	—
Unextractable[b]	1.18	0.89	0.50
$\frac{\text{AUC (metabolite)}}{\text{AUC (parent)}}$ of compartment 3 (fly extract)			
0.00	0.002	0.011	0.008
0.12	0.011	0.017	0.012
0.15	0.003	—	—
0.32	0.001	0.012	0.008
0.39	0.003	0.013	0.007

[a] Not found.
[b] Unextractable from the filter paper lining.

filter paper-bound radioactivity at 0.5 μg/fly (Table 11).

In the extractable fly (compartment 3), metabolite-to-parent ratios of 2,2′,4,4′-TCB-treated flies were highest at 5 and lowest at 0.5 μg/fly doses (Table 10). Very small amounts of metabolites of 2,2′,4,4′-TCB were extracted from the fly body, the highest ratios (0.017, 0.013) being found for metabolites (R_f = 0.12, 0.39) at 5 μg/fly. The decrease in metabolites detected in the fly body at the lowest (0.5 μg/fly) dose of 2,2′,4,4′-TCB might have been due to high covalent binding of metabolites to the fly tissues [unextractable, compartment 4, (Figs. 2–4)]. The increase in the M/P ratios at higher doses might be due to impairment of the elimination of the metabolites due to the toxicity of 2,2′,4,4′-TCB to house flies at higher doses (31). The ratios (M/P) for 3,3′,4,4′-TCB in compartment 3 were lowest, for most of the metabolites, at 5 μg/fly and highest at 0.5 μg/fly (Table 11). This again, suggests that the elimination of metabolites was impaired at lower doses.

TABLE 11

Relative Amounts of (Metabolite/Parent (M/P) of Individual Metabolites Excreted by House Flies during the 96-hr Interval Following Topical Application of 3,3',4,4'-[^{14}C]TCB and Metabolite/Parent Area under the Curve (AUC) for Fly Extracts during this Time Interval

R_f	Doses of 3,3',4,4'-[^{14}C]TBC		
	0.5 μg/fly	5 μg/fly	10 μg/fly
	M/P compartment 5 (excrement) at 96 hr		
0.00	0.47	0.32	0.19
0.12	0.09	0.03	0.03
0.17	0.11	0.04	0.04
0.22	0.08	0.06	0.04
0.34	0.33	0.22	0.15
0.43	0.41	0.30	0.18
0.50	0.12	0.06	0.10
0.70	0.13	—[a]	—
Unextractable[b]	1.15	0.65	0.24
$\frac{\text{AUC (metabolite)}}{\text{AUC (parent)}}$ *of compartment 3 (fly extract)*			
0.00	0.023	0.000	0.000
0.12	0.026	0.012	0.036
0.17	0.042	0.014	0.024
0.22	0.011	0.004	—
0.43	0.151	0.050	0.141
0.50	0.127	0.032	0.069
0.70	0.052	0.034	—

[a] Not found.
[b] Unextractable from the filter paper lining.

Environmental implication. These data confirm the role of insects in modifying environmental chemicals through commonly encountered biotransformation pathways. Numbers and relative amounts of individual metabolites vary considerably among closely related chemicals, but the patterns are very similar to those seen in mammals. Environmental accumulation of persistent lipophilic chemicals such as PCBs would be much more gradual than the topical bolus administered in these studies, so the extent of metabolism would be even greater. Significant proportions of lipophilic chemicals and metabolites are retained by insects so that food chain contributions might be predictable. In addition to direct contributions as a food source, media and other food could readily be contaminated via excrement. The large mobile biomass of insects must play a significant role in dispersion and modification of environmental contaminants and these data should be useful in refining models to include the contribution(s) by insects.

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Insecticide-Insensitive Acetylcholinesterase Can Enhance Esterase-Based Resistance in *Myzus persicae* and *Myzus nicotianae*

GRAHAM D. MOORES, GREGOR J. DEVINE, AND ALAN L. DEVONSHIRE

AFRC Institute of Arable Crops Research, Rothamsted Experimental Station, Harpenden, Herts AL5 2JQ, United Kingdom

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The acetylcholinesterase in some resistant strains of *Myzus persicae* and its extremely close relative *Myzus nicotianae* showed marked insensitivity to inhibition by the established carbamate pirimicarb (>100-fold) and by triazamate, a novel triazole aphicide (>10-fold), that acts on the same target. There was no insensitivity to a range of other carbamate and organophosphorus insecticides. This resistance mechanism appears to be rare at present and was only found in a heterozygous form associated with the commonly occurring elevated E4/FE4 esterases that confer broad cross-resistance to many aphicides. This insensitive target site mechanism, even when heterozygous, enhances the esterase-based resistance to pirimicarb and triazamate by 15- to 30-fold. © 1994 Academic Press, Inc.

INTRODUCTION

The peach–potato aphid, *Myzus persicae* (Sulzer), has evolved resistance to organophosphorus, carbamate, and pyrethroid insecticides through overproduction of a carboxylesterase (E4) that both degrades and sequesters these insecticidal esters (1). This mechanism extends to the tobacco-feeding form (2) which has been classified as a distinct species, *Myzus nicotianae,* (3), although the two can interbreed (4) and can be very difficult to distinguish morphometrically (R. L. Blackman, personal communication). For many years, this has been the only resistance mechanism identified in *Myzus* spp. (5). However, recently severe problems have occasionally been encountered in controlling aphids with carbamate insecticides and we therefore reexamined such populations for the presence of insecticide-insensitive acetylcholinesterase (AChE). This resistance mechanism is common in other homopterous species (6, 7), but we have not found it previously in *Myzus* spp. (unpublished data). However, we now report the presence of an insensitive AChE in some populations of *M. nicotianae* and *M. persicae* that confers resistance to pirimicarb and triazamate, a novel triazole aphicide acting by AChE inhibition (8).

MATERIALS AND METHODS

Aphids

The origins and rearing of the susceptible (S, clone US1L) and resistant (R_1, clone 405D; R_2, clone T1V; and Ferrara) reference clones of *M. persicae* have been described previously (9, 10). Clone 794J is a highly resistant (R_3) variant obtained from a glasshouse at Evesham in 1982. Clones 926B and 927B were established as clones from aphid strains collected from tobacco in Greece in 1990; they have been positively identified as *M. nicotianae* (4) and have FE4 levels comparable to that of E4 in 794J. Clone 1051A was collected on spinach from Okinawa; it was confirmed as *M. persicae* and has an E4 level characteristic of R_2 aphids. All identification of species was by R. L. Blackman (personal communication).

Insecticides

All insecticides used for AChE inhibition studies were of technical grade, with purities greater than 95%. For bioassay, pirimicarb was used as the commercial formula-

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tion, Aphox (50% a.i.), while triazamate and methomyl were technical grade, all diluted to the appropriate concentrations in 0.01% aqueous Agral.

AChE Preparation, Assay, and Inhibition

Approximately 250 mg of aphids were homogenized in 2 ml of 0.1 *M* phosphate buffer, pH 7.5, containing 0.1% (w/v) Triton X-100, and centrifuged at 1100*g* for 5 min. The supernatant was then passed through a Sephadex G-25 column (6 × 1-cm diameter) and then through a procainamide affinity column (11). After eluting all unbound proteins, the AChE was eluted in 20 m*M* phosphate buffer, pH 7, containing 1% Triton X-100 and 1 *M* NaCl, with a recovery of better than 80%. The overall degree of purification was not important and therefore not measured, but the removal of other esterases was confirmed by assaying the eluate with 1-naphthyl acetate (1). This separation of AChE from nonspecific carboxylesterases was essential as the large amount of E4 present in the R_3 variants can sequester significant proportions of the insecticide (1), leaving the target enzyme unexposed to inhibition and giving the appearance of AChE insensitivity *in vitro* (unpublished data).

AChE activity was assayed (12) at 25°C using a Thermomax microplate reader (Molecular Devices). Preliminary screening of AChE sensitivity to a range of insecticides (or their activated products) was determined by adding the enzyme (0.1 ml) to the wells of microtiter plates containing acetylthiocholine iodide (ATChI, 0.5 m*M*), 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB, 15 μ*M*) and various concentrations of the inhibitor to give a final volume of 0.3 ml. Activity was monitored continuously for 20 min and the mean residual activities used to estimate and compare I_{50} values. Only those inhibitors showing different potency to the AChE from various strains were studied in more detail. This same technique (6) was used with 100 μ*M* pirimicarb as a diagnostic inhibitor concentration to screen clones of *M. persicae* and *M. nicotianae*, of various resistance categories based on E4/FE4 activity, for the presence of insensitive AChE.

Inhibition was measured in the absence of substrate by mixing inhibitor in buffer with enzyme (50 μl of each) and assaying remaining activity at 10-s intervals by adding 20-μl portions to 100 μl ATChI and 100 μl DTNB to give 0.5 m*M* and 15 μ*M* final concentrations, respectively. The latter very low concentration was not rate limiting, but was necessary to avoid the inhibition of aphid AChE by DTNB (13). The bimolecular rate constants (k_i) were calculated by nonlinear regression using Enzfitter software (Leatherbarrow).

K_m values were determined at 25°C by nonlinear regression (Enzfitter) of enzyme activity measurements over 2 min with ATChI concentrations from 40 to 640 μ*M* with a minimum 10 concentrations for each.

The first-order rate constants (k_3) for reactivation of inhibited AChE were measured after incubating the enzyme with a high concentration (10^{-3} *M*) of inhibitor, removing the excess by gel filtration on Sephadex G-25 (1) and then assaying AChE activity at eight intervals between 10 and 120 min.

Bioassays

The bioassay procedure adopted for pirimicarb and methomyl was that of the FAO-recommended aphid dip test (14; alternative B) with the dipped aphids being scored for percentage death after 24 hr instead of 1 hr. This bioassay regime resulted in insufficient kill when using triazamate, so this compound was bioassayed using excised chinese cabbage leaves in "Blackman" boxes (15) with their petioles immersed in triazamate solutions for 24 hr prior to placing aphids on the leaf surface. The aphids were scored for percentage death after 24 hr exposure to the treated leaves still in triazamate solutions. LC_{50} values for both types of bioassay were calculated by probit analysis using the POLO program (Leora Software).

RESULTS AND DISCUSSION

Preliminary experiments were done using several inhibitors including pirimicarb, demeton-*S*-methyl, dichlorvos, paraoxon, omethoate, methomyl, ethiofencarb, carbofuran, triazamate, aldicarb and its sulfoxide, and sulfone, all in the presence of substrate. I_{50} plots were compared for AChE in homogenates of S, R_1, R_2, R_3, Ferrara, 927B, and 926B variants. These showed that, of the seven standard clones, only 926B had AChE with decreased sensitivity, and then only to pirimicarb (Fig. 1a) and triazamate (not shown; all other clones gave the same response as 927B and 794J). Clone 926B was therefore chosen for the more detailed kinetic work. The remainder of the inhibitors showed no differences as exemplified by Fig. 1b. It is clear from Fig. 1a that the AChE of 926B is heterogeneous, despite the clonal integrity of the cultures which was established by AChE inhibition analysis of groups of single aphids (6), so confirming that the gene is heterozygous. Since the susceptible and resistant forms of the enzyme should be present in equimolar amounts in heterozygotes (as established in house flies, 16), the plateau at 65% remaining activity indicates that the resistant component has twice the catalytic center activity (k_{cat}) of the susceptible component. This heterozygosity was again apparent in assays based on preincubation with inhibitor (Fig. 2a) rather than coincubation with substrate and inhibitor together as in the I_{50} plots. The initial rapid inhibition corresponds to the susceptible (represented by the homozygous 794J) component, and the plateau at 1.8 log % activity confirms that two-thirds of the activity is insensitive. This interpretation of the biochemical data to indicate heterozygosity of the AChE gene has since been confirmed in controlled crosses between 926B and susceptible clones (Blackman and Devonshire, unpublished). Heterogeneity of insensitive AChE in insect and mite populations is commonplace (e.g., 17, 18), typically in sexually reproducing organisms where heterozygotes will be in equilibrium with their homozygotes. The situation reported here for *Myzus* is distinctive in that asexual reproduction can perpetuate uniformly heterozygous populations.

Although methomyl appeared to be slightly more potent against the insensitive AChE in this preliminary inhibition study, k_i values later demonstrated this to be insignificant, in agreement with bioassays. This difference was perhaps due to the "protective effect" by the lower K_m of the sensitive enzymes during the coincubation (19).

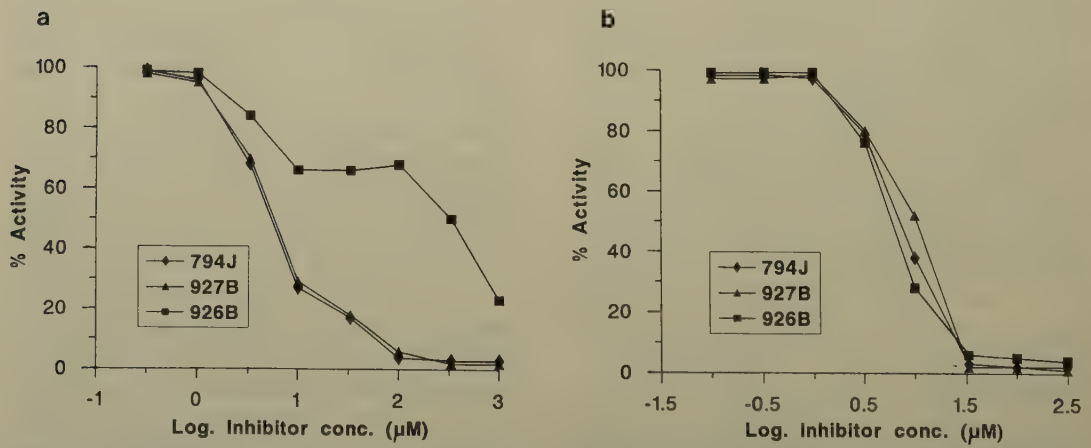

FIG. 1. *Inhibition of AChE from M. persicae (794J) and M. nicotianae (926B, 927B) by (a) pirimicarb and (b) aldicarb sulfoxide in the absence of 0.5 mM acetylthiocholine iodide. Percentage activity values are based on the mean activities monitored continuously for 20 min during these coincubation assays.*

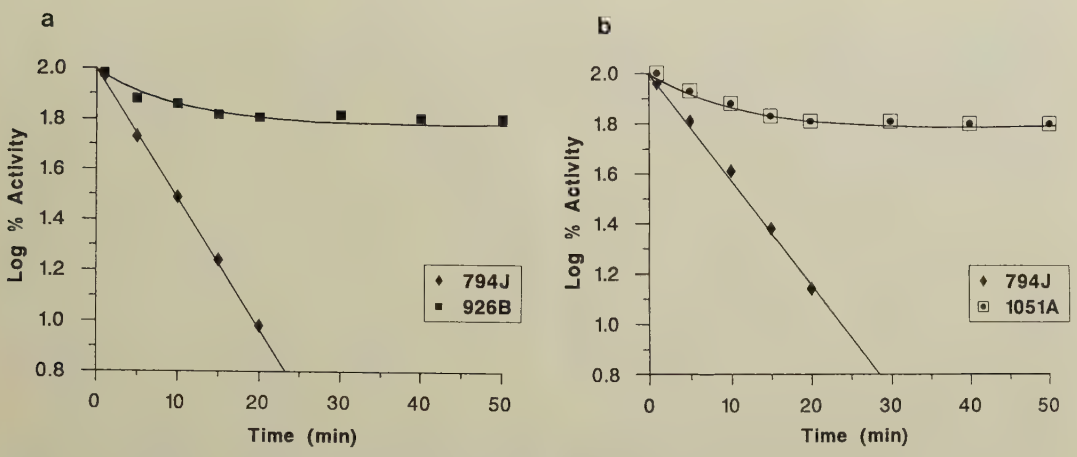

FIG. 2. *Inhibition of AChE from (a) M. persicae (794J) and Greek M. nicotianae (926B) by triazamate (1.7 μM) and (b) M. persicae from the United Kingdom (794J) and Japan (1051A) by pirimicarb (33 μM) in preincubation assays.*

The K_m of AChE from clone 926B (288 μ*M*) was twice that for any of the susceptible enzymes which did not differ significantly from each other (Table 1). Although this value represents the combined activity of both enzyme forms present in this heterozygous clone, the larger contribution is from the insensitive enzyme.

This reduced affinity for substrate was associated with a marked insensitivity of 926B AChE to pirimicarb (>100-fold) and triazamate (>10-fold), but not methomyl, compared with that of the other strains of *M. persicae* and *M. nicotianae* (Table 2). These k_i values were determined by using much higher inhibitor concentrations and shorter preincubation times than those adopted for the longer assays shown in Fig. 2. This ensured that the susceptible component was fully inhibited from the outset and that the first-order inhibition kinetics measured corresponded to the predominant insensitive enzyme. Insensitive AChE is now a common mechanism of resistance to organophosphorus and carbamate insecticides in many species, not least in the Homoptera. Insensitivity factors between one and three orders of magnitude are documented (20), in line with those reported here for *Myzus* spp.

There was no evidence for significant formation of a Michaelis complex between enzyme and inhibitor (i.e., inhibition rate remained proportional to inhibitor concentration) under the conditions used. The 10-sec

TABLE 1
K_m of AChE from Myzus Strains with Acetylthiocholine

Strain	K_m (μ*M*)[a]
USIL	141 ± 7
794J	140 ± 11
927B	115 ± 10
926B	288 ± 65

[a] Values are the means of five determinations for 926B and two determinations each for the other clones, and the standard errors were calculated from the best nonlinear regression on the combined data.

TABLE 2
Bimolecular Rate Constants (k_i, mM^{-1} min^{-1} ± SE[a]) for Inhibition of AChE from Myzus Strains

Strain	Pirimicarb	Triazamate	Methomyl
USIL	193 ± 26	7550 ± 450	69 ± 2
794J	174 ± 25	5160 ± 1530	47 ± 6
927B	134 ± 9	4360 ± 980	23 ± 5
926B	1.01 ± 0.26	554 ± 79	71 ± 4

[a] The values are the means of two or three independent determinations, and the standard errors were calculated from the best nonlinear regression on the combined data.

sampling interval of the inhibition reactions allowed accurate determination of pseudo-first-order rate constants of up to 2 min^{-1}. This would have permitted the recognition of such complex formation if the carbamylation rate constant (k_2) had been less than 5 min^{-1} (19); affinity (K_a) and k_2 constants were therefore not determined.

It is noteworthy that resistance due to this insensitive target enzyme appears to be restricted to the two compounds, pirimicarb and triazamate, that give a dimethylcarbamylated enzyme.

The susceptible enzyme showed no sign of recovering its activity by decarbamylation over a 2-hr period after removing the excess of inhibitor. Neither enzyme reactivated after inhibition by demeton-*S*-methyl. However, the insensitive enzyme from 926B did reactivate after carbamate inhibition. The k_3 value was extremely low for the monomethylcarbamylated enzyme (methomyl-inhibited), 5.74 ($\pm$1.55) $\times$ 10^{-3} hr^{-1} equivalent to a half-life of several days. The pirimicarb-inhibited enzyme reactivated more rapidly with a k_3 of 0.171 ($\pm$0.002) hr^{-1} (half-life 4 hr). Since the susceptible enzyme does not reactivate, these values are not influenced by the susceptible component in the heterozygous aphids. This reactivation of the dimethylcarbamylated AChE would serve to offset, very slightly, the resistance due to the slower inhibition (k_i) by both pirimicarb and triazamate.

The simple microtiter plate technique for characterizing insensitive AChE in individual insects (6) has been used to check many samples of *M. persicae* and *M. nicotianae* from around the world; 21 *M. persicae* from the United Kingdom, 15 from continental Europe, 16 from the United States, 1 each from Australia and China, and 3 from Japan; 9 *M. nicotianae* from Europe, 3 from the United States, and 2 from Japan. Of these, only the 3 Japanese *M. persicae* and 4 of the 9 *M. nicotianae* from Europe (all from Greece) had insensitive AChE (including the clones 926B and 1051A studied in detail). The insensitive form of AChE was only identified in *M. persicae* from Japan, originally found on spinach (clone 1051A) and cabbage; these aphids were confirmed as *M. persicae* by R. L. Blackman (personal communication). To consolidate these preliminary findings, the AChE from clone 1051A was compared with the reference strains by determination of I_{50} with pirimicarb and triazamate; Fig. 2 shows it to be indistinguishable from 926B; i.e., the gene for the insensitive enzyme in this clone is also heterozygous. For clarity, Fig. 2a shows only the data 926B with triazamate and Fig. 2b shows the data for 1051A with pirimicarb; in both cases, the data for the clone not shown coincided exactly with the inhibition curve, extrapolating back to 1.8 log % activity at zero time (i.e., 65% of the enzyme is insensitive in both clones 926B and 1051A).

The presence of the insensitive AChE in 926B confers resistance to both pirimicarb (Table 3) and triazamate (Table 4), giving resistance factors of >500, but not to methomyl (Table 5). Of this, the majority (c. 50-fold) is due to the E4 present, but the insensitive AChE enhances this at least 10-fold. Similarly, resistance in 1051A was 15- to 30-fold greater than in the R_2 standard having a corresponding level of E4, but lacking the insensitive AChE. This dramatic increase in resistance conferred by the additional mechanism, even when only

TABLE 3
Sensitivity of Aphid Clones to Pirimicarb (Aphid Dip)

Clone	R type[a] est.	AChE	LC_{50} (ppm)	95% CL	Slope	RF[b]
US1L	S	S	23	16–32	1.26	1
T1V	R_2	S	200	140–270	1.42	9
794J	R_3	S	820	670–1040	1.71	36
927B	R_3	S	1040	760–1500	1.90	45
926B	R_3	S/R	Not calc[c]	—	—	>500
1051A	R_2	S/R	2800	1920–4810	1.87	122

[a] Resistance-type based on esterase level and AChE insensitivity of clone.
[b] Resistance factor, LC_{50} for clone/LC_{50} for US1L.
[c] 50% kill unobtainable due to limiting solubility.

TABLE 4
Sensitivity of Aphid Clones to Triazamate (Systemic)

Clone	R type[a] est.	AChE	LC_{50} (ppm)	95% CL	Slope	RF[b]
US1L	S	S	0.4	0.16–1.1	1.28	1
T1V	R_2	S	2.5	0.61–6.1	1.17	6
794J	R_3	S	29	12–68	1.09	72
927B	R_3	S	21	10–42	1.16	52
926B	R_3	S/R	278	155–562	1.04	695
1051A	R_2	S/R	72	32–180	0.97	180

[a] Resistance type based on esterase level and AChE insensitivity of clone.
[b] Resistance factor, LC_{50} for clone/LC_{50} for US1L.

heterozygous, could have far-reaching effects in the control of *Myzus* spp. if it becomes widespread and severely affect the usefulness of pirimicarb, which is presently one of the most effective insecticides against aphids with the E4 mechanism only (21).

Resistance to triazamate (and pirimicarb) and R_2 (clone T1V) aphids without insensitive AChE was very similar to that recorded in an Australian population (22) and much greater than that found (23) for R_2 aphids using a dipping bioassay with a 1-hr assessment period. For aphids with R_3 level esterase only (clone 794J), resistance is much more substantial and this would render the compound ineffective against such aphids, even without any contribution from the AChE mechanism. However, triazamate has performed extremely well against *M. persicae* in field trials in the United Kingdom, apparently overcoming the widespread E4-based resistance, mainly of the R_1 and R_2 type (21). It is, therefore, unfortunate that the AChE-based resistance

TABLE 5
Sensitivity of Aphid Clones to Methomyl (Aphid Dip)

Clone	R type[a] est.	AChE	LC_{50} (ppm)	95% CL	Slope	RF[b]
US1L	S	S	37	20–69	1.13	1
794J	R_3	S	506	252–1441	1.03	14
927B	R_3	S	461	218–1442	0.92	12
926B	R_3	S/R	423	194–1127	1.11	11
1051A	R_2	S/R	215	117–443	1.14	6

[a] Resistance type based on esterase level and AChE insensitivity of clone.
[b] Resistance factor, LC_{50} for clone/LC_{50} for US1L.

mechanism new to *M. persicae* should confer such a marked increase in resistance to this novel insecticide. The dramatic effect of this gene, even when heterozygous, is not surprising; it would be expected to be genetically dominant since the insensitive AChE contributes two-thirds of the activity in the heterozygote, and insects have a large excess of AChE, which means that it must be almost fully inhibited for the insect to die (24). Clearly, to avoid selecting for this particular form of insensitive AChE, compounds to which it confers resistance should be avoided once the gene is known to be present in a population. Unfortunately, this conflicts with one of the most appropriate choices (pirimicarb) to minimize selection of aphids with resistance based on increased esterase E4 content (1).

Finally, although this work demonstrates the rarity of the insensitive AChE mechanism in *Myzus* spp., and hence the present validity of esterase-based monitoring as a positive indicator of resistance, it does underline the caution needed when dealing with insects of "known" resistance mechanisms, i.e., the reliance on monitoring with a single mechanism-specific assay for resistance and then predicting field performance of insecticides solely on this information.

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Substrate Specificity and Inhibitor Sensitivity of Cholinesterases in Homogenates of Western Flower Thrips

WEI LIU, GUANGYU ZHAO, AND CHARLES O. KNOWLES[1]

Department of Entomology, University of Missouri, Columbia, Missouri 65211

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Acetylcholinesterase (AChE) activity in homogenates of KCM strain western flower thrips, *Frankliniella occidentalis* (Pergande), was chiefly membrane bound, sensitive to eserine, and insensitive to di*iso*propyl phosphorofluoridate (DFP). The rate of substrate hydrolysis decreased in the order acetylthiocholine (ASCh)→ propionylthiocholine→ acetyl-β-methylthiocholine (MeSCh)→ butyrylthiocholine (BuSCh). Inhibition by excess substrate was not observed at concentrations up to 8.0 m*M*. Inhibition by BW 284C51 indicated the presence of two components with different sensitivity to this compound. Butyrylcholinesterase (BuChE) activity, which was considerably higher than AChE activity, was chiefly soluble and sensitive to eserine and DFP and insensitive to BW 284C51. The sulfhydryl reagents *p*-hydroxymercuribenzoic acid and 5,5′-dithio*bis*-2-nitrobenzoic acid inhibited AChE and BuChE activities. Several organophosphates and carbamates were potent inhibitors of ASCh and BuSCh hydrolysis with the organophosphates being especially active against BuSCh. Substrate specificity and inhibitor sensitivity of AChE and BuChE from UMC strain thrips were similar but not identical to that of KCM thrips. It was suggested that BuChE in western flower thrips might function as a scavenger enzyme to protect AChE from anticholinesterase insecticides. Similarities between the properties of western flower thrips cholinesterases and those reported previously for aphids were striking. © 1994 Academic Press, Inc.

INTRODUCTION

Western flower thrips (WFT),[2] *Frankliniella occidentalis* (Pergande), are major pests of numerous crops, especially some ornamentals. In addition to feeding damage by immatures and adults, WFT also transmit tomato spotted wilt virus which has an extensive host range among ornamental and vegetable species (1). Field populations of WFT have developed resistance to numerous insecticides, including some pyrethroids, organophosphates, and carbamates (2, 3). The problem is so severe that certain varieties of ornamentals are no longer grown in some Missouri greenhouses (J. M. Brown, personal communication). We have recently initiated a program to investigate the mechanisms of insecticide resistance in WFT. The target of organophosphate and carbamate insecticides is AChE (4), and insecticide-induced modifications of insect AChE have been described as resistance mechanisms (5). Therefore, to obtain fundamental information for future studies of insecticide resistance in this organism, we examined the properties of cholinesterases in two strains of WFT and compared them with those reported previously for certain other insects.

[1] To whom correspondence should be addressed.

[2] Abbreviations used: WFT, western flower thrips, *Frankliniella occidentalis* (Pergande); AChE, acetylcholinesterase (EC 3.1.1.7); BuChE, butyrylcholinesterase (EC 3.1.1.8); ASCh, acetylthiocholine iodide; PrSCh, propionylthiocholine iodide; BuSCh, butyrylthiocholine iodide; MeSCh, acetyl-β-methylthiocholine iodide; DTNB, 5,5′-dithio*bis*-2-nitrobenzoic acid; PHMB, *p*-hydroxymercuribenzoic acid sodium salt; PCMB, *p*-chloromercuribenzoic acid; BW 284C51, 1,5,-*bis*(4-allyldimethylammoniumphenyl)pentane-3-one dibromide; eserine, eserine hemisulfate; ronnoxon, *O,O*-dimethyl *O*-(2,4,5-trichlorophenyl) phosphate; Bayer 9002, *O,O*-diethyl *O*-(naphthaloximido) phosphate; diazoxon, *O,O*-diethyl *O*-(2-*iso*propyl-6-methyl-4-pyrimidinyl)phosphate; Haloxon, *O,O*-di-2-chloroethyl *O*-(3-chloro-4-methyl-2-oxo-2*H*-l-benzopyran-7-yl)phosphate; DFP, di*iso*propyl phosphorofluoridate; formetanate, formetanate HCl.

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MATERIALS AND METHODS

Insects. The KCM strain was collected in 1989 at a commercial greenhouse in western Missouri and reared on chrysanthemums in a local greenhouse maintained at 24°C during the day and 18°C during the night with a photoperiod of 12:12 (L:D) hr. Vial bioassays revealed that KCM thrips were resistant to some organophosphates, carbamates, and pyrethroids (2, 6). Beginning in October 1991, KCM thrips were sprayed with diazinon about every 45 days. Following spraying of thrips-infested chrysanthemums with diazinon, AChE and BuChE activities were rapidly depressed, and 24 days were required for their return to pretreatment levels (Knowles *et al.*, unpublished); therefore, cholinesterase studies with this strain were conducted at least 24 days after application of organophosphate. UMC strain was collected in 1992 from chrysanthemums in another greenhouse at the University of Missouri. Vial bioassays revealed low levels of resistance to some organophosphate, carbamate, and pyrethroid insecticides (Knowles *et al.*, unpublished). This strain was reared as described above, except no insecticide selection pressure was applied; soap (Safer, Safer, Inc.) was used occasionally to control aphids.

Chemicals. ASCh, PrSCh, BuSCh, MeSCh, DTNB, PHMB, Fast Blue RR salt (C.I. 37144), eserine, di*iso*propyl phosphorofluoridate (DFP), and BW 284C51 were obtained from Sigma Chemical Co. (St. Louis, MO). Diazoxon was from Ciba-Geigy Chemical Co. (Greensboro, NC). Sources for ronnoxon, dichlorvos, Bayer 9002, Haloxon, formetanate, methomyl, and oxamyl were given by Errampalli and Knowles (7).

Enzyme assays and inhibition. For assay of cholinesterases, adult WFT selected without regard to sex or age were homogenized for 15 sec in ice-cold 20 m*M* phosphate buffer (pH 7.6). The homogenate was centrifuged at 450*g* for 10 min, and the supernatant was centrifuged at 110,000*g* for 1 hr. The pellet was resuspended to original volume in the homogenization buffer. The 450*g* supernatant and the 110,000*g* supernatant and pellet were used as enzyme sources. One WFT weighed about 57 μg (wet wt) and contained 8.17 ± 0.57 μg protein as determined by the Miller (8) modification of the Lowry method with bovine serum albumin (Sigma) as standard. The 450*g* supernatant, 110,000*g* supernatant, and 110,000*g* pellet contained about 8.0, 5.61, and 2.39 μg protein per thrips, respectively. Cholinesterase activity was measured in WFT homogenates immediately after preparation by the Gorun *et al.* (9) modification of the method of Ellman *et al.* (10). Preliminary experiments conducted with the 450*g* supernatant of KCM thrips revealed that hydrolysis of ASCh (1.0 m*M*) was linear with an incubation time of up to 60 min using the equivalent of six thrips per assay and with a homogenate concentration of up to eight thrips per assay when incubated for 15 min. Hydrolysis of BuSCh (1.0 m*M*) was linear with a homogenate concentration of up to four thrips per assay when incubated for 5 min. The reaction mixture consisting of 160 μl of enzyme preparation equivalent to six thrips (ASCh, MeSCh) or 1.5 thrips (PrSCh, BuSCh) and 40 μl of substrate (0.1 to 8.0 m*M*, usually 1.0 m*M* final) was incubated in a water bath at 37.5°C for 15 min (ASCh, MeSCh) or 5 min (PrSCh, BuSCh) prior to adding 1.8 ml of DTNB reagent. The absorbance was read immediately at 412 nm (Milton Roy Spectronic 1201), and 13,600 M^{-1} cm^{-1} was used as the extinction coefficient of the thionitrobenzoate ion (9). Using this static modification of the original kinetic method was necessary because DTNB was found to have an inhibitory effect on WFT cholinesterases. Corrections for nonenzymatic degradation of substrate and for endogenous homogenate interference were made when appropriate.

To study inhibition of cholinesterases, aliquots of potential inhibitors dissolved in distilled water or acetone at appropriate

concentrations were added to the tubes. (When acetone was used it was allowed to evaporate before adding the enzyme preparation.) Inhibitor and enzyme preparation were incubated for 15 min at 25°C prior to adding the substrate.

Electrophoresis studies. Thrips were homogenized in 0.1 *M* phosphate buffer (pH 6.5). The homogenate was centrifuged at 450*g* for 10 min, and aliquots of the supernatant, which had a protein concentration between 20 and 40 μg/ml, were used. Electrophoresis was conducted with the PhastSystem (Pharmacia, Piscataway, NJ). Native gradient (8–25%) polyacrylamide PhastGels and native buffer strips consisting of 0.88 *M* L-alanine and 0.25 *M* Tris at pH 8.8 were used. The effects on ASCh hydrolyzing activity of various concentrations of sodium deoxycholate and Triton X-100 in sample buffer, running buffer, and/or gel were examined. Electrophoresis was carried out at 400 V and 10 mA (160 vhr, Pharmacia) at 15°C. After electrophoresis, gels were cut into strips and placed in 0.1 *M* phosphate buffer (pH 6.5; controls) or in buffer solutions of BW 284C51 (100 μ*M*), eserine (100 μ*M*), DFP (1.0 μ*M*), or diazoxon (1.0 μ*M*). After 30 min, gels were rinsed with distilled water and stained for cholinesterase activity. Hydrolysis of acylthiocholines and MeSCh was detected by the method of Karnovsky and Roots (11) as modified by Bunyan and Taylor (12). Gels were destained in 7.0% acetic acid.

RESULTS

Distribution studies. The distribution of ASCh and BuSCh hydrolyzing activities in centrifugal fractions of KCM thrips is given in Table 1. The 450*g* supernatant was very active toward both substrates. Centrifugation of the 450*g* supernatant at 110,000*g* yielded a pellet and a supernatant with about 71% of the total activity toward ASCh and BuSCh, respectively. This indicated that the enzyme(s) hydrolyzing ASCh was chiefly membrane bound, whereas the enzyme(s) hydrolyzing BuSCh was chiefly soluble. BuSCh was hydrolyzed appreciably faster than ASCh by each of the preparations of KCM thrips. The centrifugal distribution and relative rates of hydrolysis of ASCh and BuSCh by preparations of UMC thrips were similar to those of KCM thrips (Table 1).

Studies with selective inhibitors and substrates. The enzymatic degradation of acylthiocholines and MeSCh at a final concentration of 1.0 m*M* was sensitive to eserine (Table 2), a selective inhibitor of cholinesterases (13, 14). This carbamate at a concentration of 100 μ*M* gave greater than 95% inhibition of the hydrolysis of ASCh, PrSCh, BuSCh, and MeSCh by the 450*g* supernatants of KCM and UMC thrips, and at 10 μ*M* it yielded complete inhibition of the hydrolysis of ASCh by the 110,000*g* pellet fraction and of BuSCh by the 110,000*g* supernatant and pellet frac-

TABLE 1

Distribution of Acetylthiocholine- and Butyrylthiocholine-Hydrolyzing Activities in Centrifugal Fractions of KCM Western Flower Thrips Homogenates[a]

Fraction	ASCh		BuSCh	
	Relative activity (%)	Specific activity (nmol/mg protein/min)	Relative activity (%)	Specific activity (nmol/mg protein/min)
Whole homogenate	100	59.52 (5.72)	100	766.85 (60.11)
450*g* Supernatant	95.8 (11.4)	58.45 (6.96)	96.5 (10.8)	758.57 (84.89)
110,000*g* Supernatant	24.8 (7.97)	22.42 (7.21)	70.9 (9.40)	825.98 (109.51)
110,000*g* Pellet	71.0 (7.97)	132.87 (14.95)	25.6 (9.40)	618.33 (227.19)

[a] Data are means (±SD); $n = 15$. With UMC thrips the rates (nmol/mg protein/min) of ASCh and BuSCh hydrolysis were 127.82 (2.83) and 721.83 (139.24) for the 110,000*g* pellet and supernatant, respectively; $n = 3$.

TABLE 2

Potency and Selectivity of Organophosphates and Carbamates as Inhibitors of Hydrolysis of Acetylthiocholine and Butyrylthiocholine by Homogenates of KCM and UMC Western Flower Thrips

	pI_{50} (mol/liter)[a]					
	KCM			UMC		
Inhibitor	ASCh (450*g* S)	ASCh (110,000*g* P)	BuSCh (110,000*g* S)	ASCh (450*g* S)	Selectivity BuSCh-ASCh[b]	Interstrain selectivity[c]
Ronnoxon	6.05	5.72	7.98	6.47	181.97	2.63
Dichlorvos	7.10	7.08	~10.37	7.88	1,949.84	6.03
Haloxon	5.95	5.83	~9.99	5.23	14,454.40	(5.25)
Bayer 9002[d]	6.56	7.42	~12.25	7.21	67,608.30	4.47
DFP	3.95	5.00	~10.25	3.78	177,827.94	(1.48)
Eserine[e]	7.19	7.42	6.88	6.77	(3.47)	(2.63)
Formetanate	4.58	4.78	4.47	4.76	(2.04)	1.51
Methomyl	5.63	5.65	5.80	5.70	1.41	1.17
Oxamyl	6.56	6.17	7.02	7.31	7.08	5.62

[a] pI_{50} Dependent upon the use of inhibitor concentrations < 1.0 n*M* for their calculation are designated as approximate because inhibitor concentration may not have exceeded enzyme concentration by 10-fold.

[b] Selectivity is between 110,000*g* supernatant with BuSCh and 110,000*g* pellet with ASCh using KCM thrips. Parentheses indicate selectivity in favor of ASCh.

[c] Selectivity is between 450*g* supernatants with ASCh using KCM and UMC strains, respectively. Parentheses indicate selectivity in favor of KCM thrips.

[d] Bayer 9002 gave a pI_{50} of ~10.96 with 110,000*g* pellet of KCM thrips with BuSCh.

[e] Eserine gave pI_{50}s of 6.61, 6.59, and 7.07 with the 450*g* supernatant of KCM thrips with PrSCh, BuSCh, and MeSCh, respectively, and 6.75 for the 110,000*g* pellet of KCM thrips. Eserine gave pI_{50}s of 6.42, 5.87, and 6.77 with the supernatant of UMC thrips with PrSCh, BuSCh, and MeSCh, respectively.

tions of KCM thrips (data not shown). These results indicated that the enzymatic degradation of acylthiocholines and MeSCh at a concentration of 1.0 m*M* by WFT was catalyzed by cholinesterases with little, if any, contribution from other esterases.

Enzymatic hydrolysis of ASCh but not BuSCh was inhibited by BW 284C51 (Fig. 1), a selective reversible inhibitor of AChE's (15). With maximum inhibition of ASCh hydrolysis of only 75–80% of control at 1.0 m*M*, this compound was not as potent as was expected based on previous studies with BW 284C51 and other insect

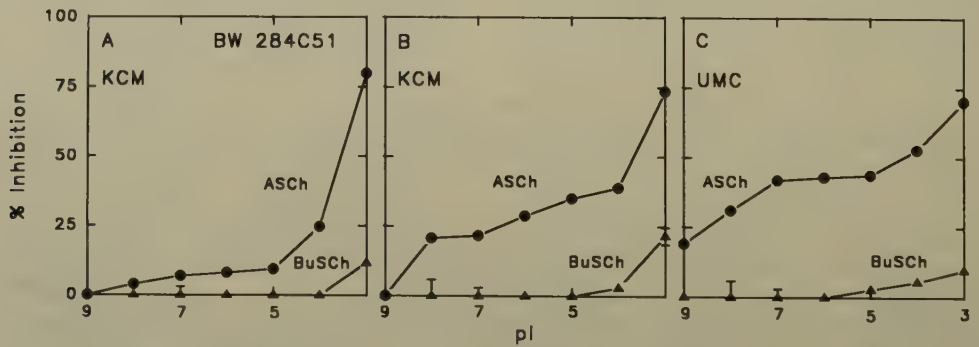

FIG. 1. *Effect of BW 284C51 concentration on hydrolysis of acetylthiocholine and butyrylthiocholine (1.0 m*M*) by preparations of western flower thrips. Data are means of at least three replicates; SD value is given when greater than size of symbol. (A) Supernatant (450g) of KCM thrips. (B) Pellet (110,000g) of KCM thrips. BW 284C51 at 1.0 m*M *gave only 30% inhibition of the hydrolysis of BuSCh by the 110,000g supernatant of KCM thrips (data not shown). (C) Supernatant (450g) of UMC thrips.*

AChE's (14). However, inspection of the inhibition curves revealed two components with different sensitivities to BW 284C51 present in the 450*g* supernatant and the 110,000*g* pellet from KCM thrips and in the 450*g* supernatant from UMC thrips. The apparent pI_{50} for BW 284C51 and the more sensitive component were about 6.5, whereas that for the more resistant component was about 4.5. Moreover, the relative activities of the two enzymes appeared to differ in the various preparations with the activity of the sensitive component being higher in the 450*g* supernatant from UMC thrips than in that from KCM thrips.

DFP, a selective irreversible inhibitor of BuChE's (16), was remarkably selective in its inhibition of the hydrolysis of ASCh and BuSCh (Fig. 2). At a concentration of 100 n*M*, DFP yielded complete inhibition of the hydrolysis of BuSCh and no inhibition of the hydrolysis of ASCh with the 450*g* supernatants from KCM and UMC thrips. With the 110,000*g* pellet from KCM thrips, DFP at 10 n*M* gave greater than 90% inhibition of BuSCh hydrolysis and less than 5% inhibition of ASCh hydrolysis, and with the 110,000*g* supernatant DFP gave greater than 90% inhibition of BuSCh hydrolysis. At high concentrations, DFP also inhibited ASCh hydrolysis, but 15–25% of the activity toward this substrate was not inhibited

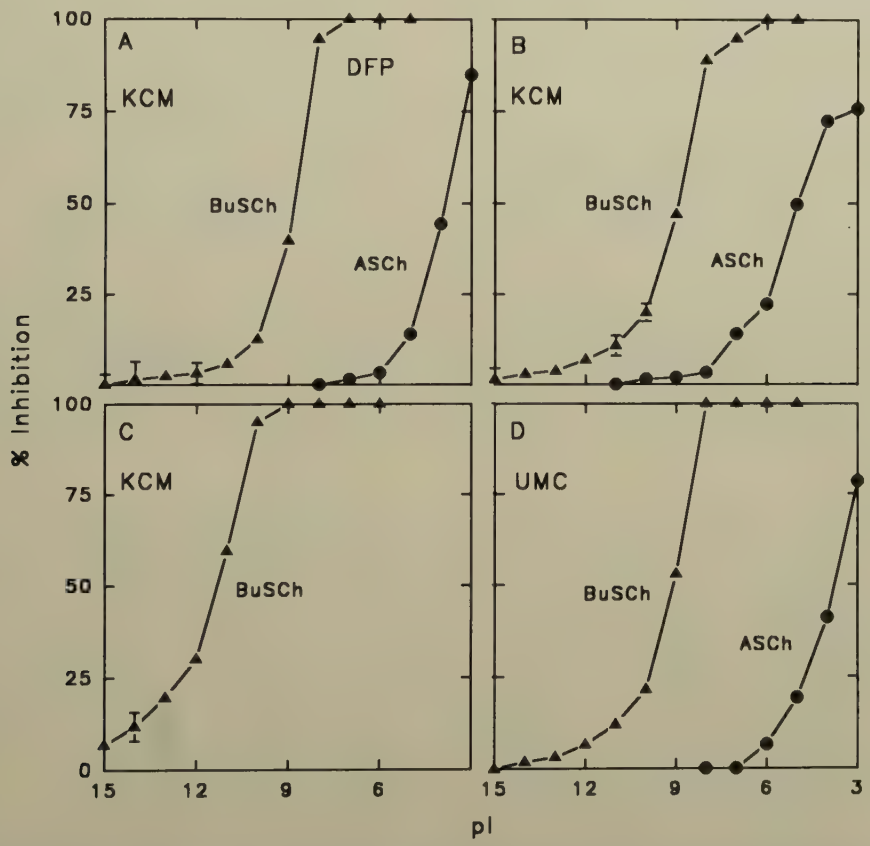

FIG. 2. *Effect of DFP concentration on hydrolysis of acetylthiocholine and butyrylthiocholine (1.0 mM) by preparations of western flower thrips. Data are means of at least three replicates; SD value is given when greater than size of symbol. (A) Supernatant (450g) of KCM thrips.* pI_{50}*s were 3.95 and 9.00 for ASCh and BuSCh, respectively. (B) Pellet (110,000g) of KCM thrips.* pI_{50}*s were 5.00 and 9.10 for ASCh and BuSCh, respectively. (C) Supernatant (110,000g) of KCM thrips.* pI_{50} *was ~10.25 for BuSCh. (D) Supernatant (450g) for UMC thrips.* pI_{50}*s were 3.78 and 9.08 for ASCh and BuSCh, respectively.*

by the organophosphate at 1.0 m*M*. These results with DFP suggested that the respective enzymes hydrolyzing ASCh and BuSCh were relatively specific with regard to these two substrates.

The effects of PHMB and DTNB, two sulfhydryl inhibitors, on ASCh and BuSCh hydrolysis by preparations of KCM thrips are presented in Fig. 3. With the 450*g* supernatant, PHMB and DTNB inhibited the hydrolysis of ASCh with the former being more potent than the latter. PHMB also inhibited the hydrolysis of ASCh by the 110,000*g* pellet, and the curve was similar to that observed with the 450*g* supernatant. With the 450 and 110,000*g* supernatants and BuSCh, low concentrations of PHMB and DTNB (tested on the 450*g* supernatant only) occasionally were stimulatory (10% or less), whereas high concentrations were inhibitory.

Activity–substrate concentration data from KCM thrips revealed that hydrolysis rates decreased in the order BuSCh→ PrSCh→ ASCh→ MeSCh by the 450 and 110,000*g* supernatants and by the 110,000*g* pellet (Fig. 4). A similar pattern of activity was observed with the 450*g* supernatant of UMC thrips (data not shown). In the presence of 10 n*M* DFP, hydrolysis rates decreased in the order ASCh→ PrSCh→ MeSCh→ BuSCh by the 110,000*g* pellet; however, PrSCh was hydrolyzed faster than the other substrates at high concentrations (Fig. 4). Since BuChE activity was inhibited by DFP, the hydrolysis of the thiocholine esters and MeSCh, particularly at 1.0 m*M*, observed in the activity–substrate concentration experiment was due mainly to AChE. In the presence of DFP, hydrolysis of 1.0 m*M* ASCh, PrSCh, BuSCh, and MeSCh was 110.46, 22.22, 5.82, and 75.39% of that observed in the absence of this organophosphate. Thus, relative rates of hydrolysis by AChE in the 110,000*g* pellet were 100 for ASCh, 20 for PrSCh, 5 for BuSCh, and 68 for MeSCh. Excess substrate inhibition was not observed in the absence or presence of DFP.

Electrophoretic studies. Electrophoretic experiments were conducted with the 450*g* supernatants of KCM and UMC thrips (Fig. 5). After overnight staining, activity toward ASCh was localized at the origin; it remained at the origin in the presence of 0.1% sodium deoxycholate. However, in the presence of 0.5% Triton X-100, the protein(s) entered the gel, but the ASCh hydrolyzing activity was presented as a smear. Under these conditions it was not possible to determine with certainty the number of enzymes acting on this substrate. Whether

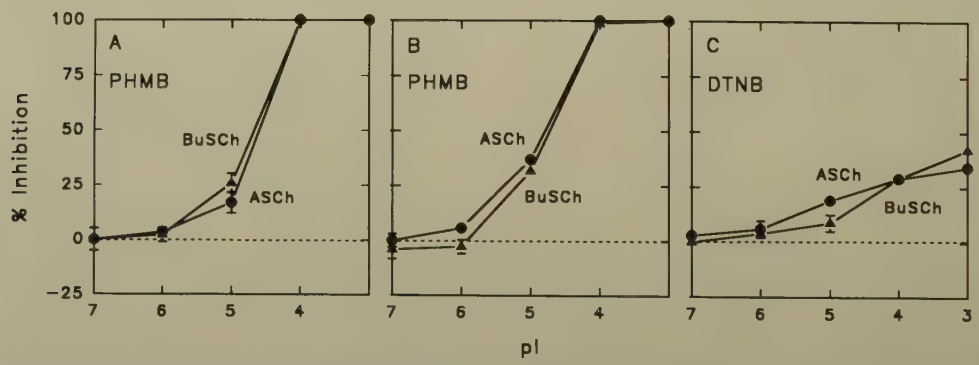

FIG. 3. *Effect of* p-*hydroxymercuribenzoic acid and 5,5′-dithio*bis-*2-nitrobenzoic acid concentrations on hydrolysis of acetylthiocholine and butyrylthiocholine (1.0 m*M*) by preparations of KCM strain western flower thrips. Data are means at least of three replicates; SD value is given when greater than size of symbol. (A) PHMB and 450*g* supernatant. pI_{50}*s were 4.59 for ASCh and 4.69 for BuSCh. (B) PHMB and 110,000*g* pellet (ASCh) and supernatant (BuSCh). pI_{50}*s were 4.83 for ASCh and 4.72 for BuSCh. (C) DTNB and 450*g* supernatant of KCM thrips.*

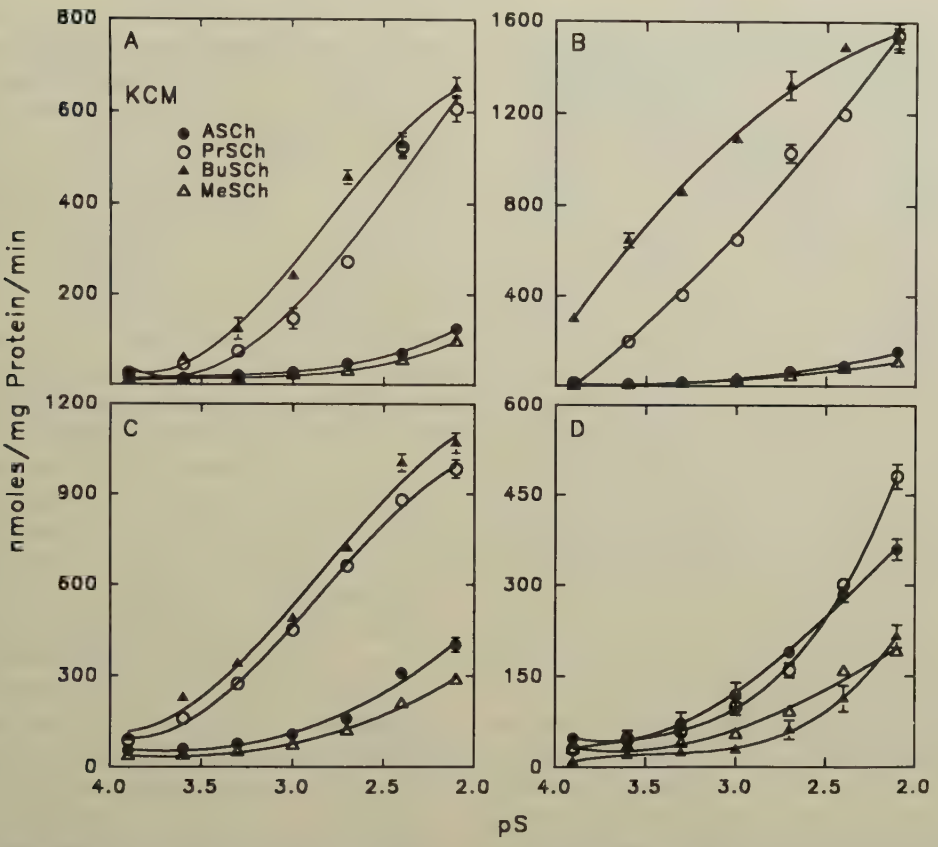

FIG. 4. *Effect of varying substrate concentrations on hydrolysis of acylthiocholines and acetyl-β-methylthiocholine by preparations of KCM strain western flower thrips. pS, negative logarithm of substrate concentration in mol/liter. Data are means of at least three replicates; SD value is given when greater than size of symbol. (A) 450g Supernatant. (B) 110,000g Supernatant. (C) 110,000g Pellet. (D) Pellet (110,000g) in presence of 10 mM DFP.*

at the origin or in the gel, eserine, BW 284C51, and diazoxon abolished activity toward ASCh, but DFP had little discernible effect. With BuSCh as substrate, two bands of intense activity were observed after only 50 min of staining. Two less intense bands were sometimes observed; one was slightly above, the other slightly below the two major bands. The protein(s) catalyzing the hydrolysis of BuSCh migrated between the boundary established by the 132K dimer and 66K monomer of the bovine serum albumin markers. No hydrolysis of BuSCh was apparent at the origin even after staining overnight. Hydrolysis of BuSCh was inhibited by eserine, DFP, and diazoxon but not by BW 284C51. With MeSCh and overnight staining, only hydrolytic activity at the origin was observed, and the response to the inhibitors was similar to that toward ASCh. With PrSCh as substrate and overnight staining, moderately intense activity was observed at the origin that responded to the inhibitors similar to activity toward ASCh and MeSCh. Two other bands of PrSCh activity were present that corresponded to the two major bands of BuSCh activity and that responded to the inhibitors as did the activity against BuSCh.

Organophosphates and carbamates as inhibitors of cholinesterases. When comparisons were made among organophosphates as inhibitors of ASCh hydrolysis by the 110,000*g* pellet and BuSCh hydrolysis

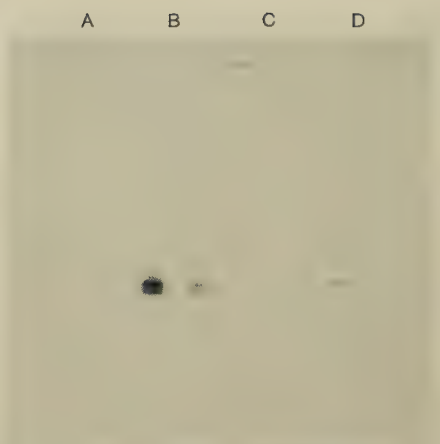

FIG. 5. *Nondenaturing polyacrylamide gel electrophoresis of two different concentrations (about 5.0 and 1.25 μg total protein) of 450g supernatant of KCM thrips. Staining: (A) ASCh; (B) BuSCh; (C) MeSCh; and (D) PrSCh. Patterns with 450g supernatant of UMC thrips were similar to those of KCM thrips.*

by the 110,000*g* supernatant, it was apparent that they were appreciably more potent against BuSCh (Table 2). Further, selectivity increased as the electron-donating properties of the alkyl substituents increased. Thus, the least selective were the dimethyl-substituted compounds (ronnoxon and dichlorvos) followed in order of increasing selectivity by di-2-chloroethyl (Haloxon), diethyl (Bayer 9002), and DFP. These organophosphates gave complete inhibition of BuSCh hydrolysis with concentrations for 100% inhibition ranging from 1.0 m*M* to 1.0 μ*M*. With ASCh hydrolysis by the 110,000*g* pellet, none of the organophosphates gave complete inhibition at 1.0 m*M*, the highest concentration tested, except for dichlorvos which yielded 100% inhibition at 10 μ*M*. The four carbamates were much less selective as inhibitors of ASCh and BuSCh hydrolysis when compared with the organophosphates (Table 2).

In terms of interstrain selectivity as assayed with 450*g* supernatants and ASCh, UMC thrips were somewhat more sensitive to ronnoxon, dichlorvos, and Bayer 9002, while KCM thrips were more sensitive to Haloxon (Table 2). DFP was not selective. With regard to the carbamates, UMC thrips were more sensitive than KCM thrips to oxamyl, and KCM thrips were more sensitive than UMC thrips to eserine. Formetanate and methomyl exhibited little selectivity between the two strains.

DISCUSSION

Based on accepted criteria (13), homogenates of WFT contained both AChE and BuChE activities, although these enzymes possessed at least one property that is very unusual for cholinesterases that will be discussed below, namely inhibition by sulfhydryl reagents. AChE was present in WFT homogenates because the eserine-sensitive, DFP-insensitive activity hydrolyzed acylthiocholine esters in the order ASCh→ PrSCh→ BuSCh. MeSCh was hydrolyzed in the presence and absence of DFP. Experiments with BW 284C51 indicated the presence of two components with differing sensitivities to this compound. Whether this heterogeneous response to BW 284C51 is due to heterogeneity of the cultures or of the enzyme in every individual is unknown. Currently the sensitive component is thought to be similar to the AChE present in susceptible WFT, and the relatively insensitive component is thought to have resulted from selection with anticholinesterase insecticides. In support of this view is the fact that the activity of the insensitive component appeared to be higher in diazinon-selected KCM thrips than in UMC thrips. The electrophoresis studies clearly indicated the presence of AChE in KCM and UMC thrips but unfortunately did not add to our knowledge of multiple forms. However, research on the mechanisms of diazinon resistance in WFT is in progress and may contribute to our understanding of the significance of these components.

Hydrolysis of acylthiocholines and MeSCh did not exhibit excess substrate inhibition at concentrations up to 8.0 m*M*. It is possible that excess substrate inhibition is not characteristic of WFT AChE. Although substrate inhibition is typical of many insect AChE's, some aphid AChE's did not

exhibit this phenomenon (14, 17). Alternatively the apparent absence of excess substrate inhibition may have been due to the presence of the two AChE components. Each of the two components may have a different p*S* optimum for ASCh, and our assay of the mixture did not result in their resolution. Nolan and Schnitzerling (18), working with AChE of an organophosphate-resistant strain of the cattle tick, *Boophilus microplus* (Canestrini), observed two components, an organophosphate-insensitive component with a p*S* optimum for ASCh of 0.27 m*M* and a sensitive component with an optimum p*S* of 10 m*M*. Less likely is the possibility that the substrate inhibition was masked by the presence of a nonspecific esterase(s) hydrolyzing ASCh at high concentrations as was reported for the two-spotted spider mite, *Tetranychus urticae* Koch (19).

BuChE also was present in WFT because of the eserine-sensitive, DFP-sensitive hydrolysis of BuSCh. Activity toward this substrate was highest in the 110,000*g* supernatant; however, appreciable activity toward BuSCh also was found in the 110,000*g* pellet. Although the nature of the BuSCh activity in the 110,000*g* pellet was not studied in detail, it is noteworthy that when compared with the activity against this substrate in the 110,000*g* supernatant, BuSCh hydrolysis was less sensitive to DFP, Bayer 9002, and eserine by 14.12, 19.50, and 1.35 times, respectively. This does not necessarily indicate that the BuSCh activities in the two fractions were results of different enzymes because AChE activity, which was shown to be present in the pellet, also hydrolyzed BuSCh to some extent and was less sensitive than BuChE activity to DFP and Bayer 9002. Electrophoresis studies indicated the presence of two electromorphs with intense activity toward BuSCh but no apparent activity toward ASCh and MeSCh and whose activities were inhibited by eserine, DFP, and diazoxon, but not by BW 284C51. However, it is unlikely that one electromorph corresponded to the BuSCh activity in the 110,000*g* supernatant and the other to the activity in the pellet.

The 450*g* supernatant of KCM WFT hydrolyzed ASCh and BuSCh at the rates of 7.96 and 103.27 μmol/min/g (wet wt), respectively. Novozhilov *et al.* (20), in their study of cholinesterases in whole homogenates of 35 species in the Order Homoptera, including 30 species of aphids, detected ASCh hydrolysis in all organisms and BuSCh hydrolysis, which they attributed to BuChE, in 16 of 33 species. The fastest hydrolysis of ASCh was by the currant aphid, *Cryptomyzus ribis* (L.), which degraded the substrate at the rate of 11.0 μmol/min/g (wet wt), followed by the green peach aphid, *Myzus persicae* (Sulzer), at 5.4 μmol/min/g. Thus, ASCh hydrolysis by KCM WFT occurred at a rate between the two most active aphid species. Hydrolysis of BuSCh by aphid preparations was never faster than that of ASCh, and the maximum rate was 3.7 μmol/min/g by the green peach aphid. Thus, BuSCh hydrolysis was about 12.98 times faster than that of ASCh by KCM WFT and about 27.91 times that of the green peach aphid.

Working with WFT, we found that AChE and BuChE activities were inhibited by PHMB and DTNB. PHMB was more potent than DTNB and yielded complete inhibition of ASCh and BuSCh hydrolysis at a concentration of 0.1 m*M*. Moreover, similarities in the inhibition curves for ASCh and BuSCh, particularly by PHMB, indicated that the enzymes hydrolyzing these two substrates were closely related. BuChE activity was occasionally activated slightly (10% or less) by low concentrations of PHMB and DTNB. AChE and BuChE activities of vertebrates and insects typically are not affected by sulfhydryl reagents (14). Exceptions to this generalization have been found in some insects in the Order Homoptera (17, 20–25). For example, Novozhilov *et al.* (20) found that ASCh-hydrolyzing activity in all 29 species of aphids tested in the Suborder Aphidinea was inhibited (range 3 to 84%) when prein-

cubated with 0.77 m*M* DTNB for 10 min. In the Suborder Aleyrodinea, DTNB was shown to inhibit ASCh hydrolysis by homogenates of the tobacco whitefly, *Bemisia tabaci* (Gennadius) (25), but not by the greenhouse whitefly, *Trialeurodes vaporariorum* (Westwood) (20). The inhibitory effect on aphid AChE was explained by the presence of a cysteine residue that reacted covalently with the sulfhydryl reagents blocking hydrolysis of the substrate (21). Thus, it is suggested that cysteine residues likely are present at or near the active site(s) of WFT AChE and BuChE.

In a recent review, Toutant (14) indicated that of AChE and BuChE only AChE had been convincingly demonstrated in insects. This conclusion appeared to be based mainly on the fact that BuChE's reported previously from the spirea aphid, *Aphis citricola* van der Goot, and the spring grain aphid, *Schizaphis gramina* (Rondani), were active only at high substrate concentrations and were resistant to inhibition by certain organophosphates (17, 24, 26), two properties that were not characteristic of BuChE in WFT. However, our finding that AChE and BuChE activities in WFT were sensitive to sulfhydryl reagents, a property presently thought to be characteristic of some noncholinesterases (14), may call into question their homology with vertebrate and perhaps some insect cholinesterases.

It is obvious that some of the properties of AChE's and BuChE's of WFT and aphids were similar and unlike those of other insect cholinesterases. Although thrips are in the Order Thysanoptera, they are closely related to aphids phylogenetically (27). These biochemical similarities between WFT and aphid cholinesterases may indicate that they are even more closely related than previously thought.

A few comments regarding the function of AChE's and BuChE's in WFT seem appropriate. AChE has been known for some time to be involved in facilitating cholinergic transmission in the insect central nervous system by hydrolyzing the neurotransmitter acetylcholine. Thus, its presence in active insects like WFT was expected. However, the presence of such high levels of BuChE activity was not anticipated. The precise function(s) of BuChE in vertebrates and certainly in insects remains unknown. However, it was observed that some humans with defective BuChE genes resulted in reduced levels of serum BuChE activity were especially sensitive to some anticholinesterase insecticides, and it was suggested that the BuChE might function as a scavenger enzyme to remove those compounds, thus preventing them from reaching the AChE (28). It is suggested that in WFT the BuChE could also function as a scavenger enzyme, affording some protection of thrips to anticholinesterase insecticides.

ACKNOWLEDGMENTS

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PESTICIDE BIOCHEMISTRY AND PHYSIOLOGY 49, 132–137 (1994)

Partial Purification and Characterization of a Greenbug (Homoptera: Aphididae) Esterase Associated with Resistance to Parathion

BLAIR D. SIEGFRIED AND ANTHONY J. ZERA

Department of Entomology, 202 Plant Industry Building, and School of Biological Sciences, 113 Lyman Hall, University of Nebraska, Lincoln, Nebraska 68583

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Esterases from the greenbug associated with resistance to organophosphate insecticides were partially purified from both resistant and susceptible strains by ion-exchange chromatography and preparative electrophoresis. Enzymes from both strains exhibited similar elution profiles although the activity peak from the resistant strain was greater in both height and total area. The dominant esterase isozymes from both resistant and susceptible strains were further purified by preparative native electrophoresis. Kinetic analysis of the resulting enzyme indicated that the K_m of the esterase was identical for the two strains, although V_{max} was consistently three- five-fold higher in the resistant strain. Inhibition of the activity from both strains by paraoxon was also identical, providing further support for the presence of the same isozyme in the two strains. These results suggest that resistance is associated with overproduction of an isozyme present in the susceptible strain rather than the presence of an enzyme with altered properties. © 1994 Academic Press, Inc.

INTRODUCTION

The greenbug, *Schizaphis graminum* (Rondani), is a worldwide pest of small grains and sorghum. In the United States, organophosphate (OP) insecticides, such as parathion and disulfoton, are commonly used for greenbug management programs. Widespread resistance of the greenbug to the systemic OP, disulfoton, in the mid-1970s resulted in increased application rates and an eventual rotation to other insecticides (1, 2). The introduction of new sorghum cultivars resistant to the greenbug during this period reduced the extent and economic importance of the resistance problem. However, insecticides continue to play a major role in controlling economically damaging populations of greenbugs, and the potential for resistance development remains high. This point is emphasized by the recent observation of resistance development in certain areas of Kansas where control failures have been reported (3).

Studies into the mechanisms of insecticide resistance have shown that high levels of general esterase activity, measured both with model substrates and electrophoretically, are present in greenbugs collected from areas of control failures (3–5). Elevated esterase activity, based on polyacrylamide gel electrophoresis and staining with α-naphthyl acetate, has been correlated with resistance, although two different patterns of esterase isozymes have been detected. Type I aphids exhibit a single esterase band that is either absent in susceptible greenbugs or expressed at levels below the limits of detection. Type II aphids exhibit multiple, darkly stained esterase bands relative to the susceptible strain, although a single esterase band predominates.

Recent investigations have provided more conclusive evidence that elevated esterase activity is partially responsible for resistance in both greenbug strains (4–6). *In vivo* and *in vitro* metabolism studies have shown that esterases present within the resistant strains are not active toward parathion, but are active toward paraoxon, the oxidized and active form of the parent molecule. In addition, these enzymes do not catalyze true hydrolysis of the insecticidal ester, but rather tightly bind to the insecticide, thus sequestering it away from the tar-

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get site. Resistance apparently results from increased levels of esterases capable of binding higher numbers of insecticide molecules relative to the susceptible strain.

Although the role of these esterases has been clearly established in the resistance mechanisms, the genetic and biochemical regulation of elevated activity remains uncertain. There are two possible genetic changes that could lead to the observed esterase-mediated resistance. The first is a stable, heritable change in the DNA sequence leading to a change in the amino acid sequence and giving rise to a protein with altered properties. Alternatively, a change in the sequence of regulatory DNA outside the protein coding region or multiple copies of the same gene (gene amplification) would give rise to increased mRNA and protein synthesis. In this paper, we report the partial purification and properties of the Type II greenbug esterase from resistant and susceptible greenbugs and suggest a possible molecular basis of resistance in this strain.

MATERIALS AND METHODS

Greenbug strains. The parathion-resistant greenbug strain was established during August 1988 with greenbugs collected from a sorghum field in Sherman County, Texas, where control failure with organophosphate insecticides was reported and where at least one application of Lorsban was recorded. A colony of the resistant greenbugs was provided by Ms. Roxanne Shufran, Kansas State University, and established at the University of Nebraska in October 1991. The susceptible greenbug strain was collected from sorghum fields in Lancaster County, Nebraska, and established in the laboratory during August 1990. Preliminary bioassays indicated susceptibility to parathion and disyston. Both aphid strains were reared on sorghum (*Sorghum bicolor* (L.) Moench) seedlings (Funks G-522A) at 26°C with a 16:8 (L:D) photoperiod. Bioassays of the resistant and susceptible strains indicated a resistance level of approximately 30-fold to parathion (5).

Enzyme assays. During purification, esterase activity was measured according to the technique of van Asperen (7) by measuring the production of α-naphthol from α-naphthyl butyrate as substrate. The reaction mixture consisted of phosphate buffer (0.02 *M* sodium phosphate, pH 7.0) and 0.25 m*M* α-naphthyl butyrate and was incubated for 15 min at 30°C. The reaction was stopped and color was developed by the addition of 166 μl 0.3% diazo blue B in 3.5% sodium lauryl sulfate. The absorbance was recorded 15 min later against an enzyme blank, and the concentration of substrate hydrolyzed was determined from a standard curve of α-naphthol. For inhibition experiments, esterase activity was determined by following the production of *p*-nitrophenol from *p*-nitrophenyl acetate. The 1-ml reaction mixture consisted of 0.02 *M* sodium phosphate, 0.5 m*M* *p*-nitrophenyl acetate, and 10 μl of enzyme preparation. The reaction was initiated by the addition of substrate, and the change in absorbance was recorded at 400 nm.

Continuous native PAGE. Continuous native polyacrylamide gel electrophoresis was performed in a Bio-Rad (Hercules, CA) Mini-Protean II vertical unit, using a 7.5% acrylamide gel with a continuous Tris–glycine buffer system (50 m*M*, pH 8.3). Samples were diluted 1:1 with 50 m*M* Tris–glycine buffer (pH 8.3) with 20% sucrose (w/v). Individual wells were loaded with equal amounts of protein, and 0.1% bromophenol Blue in 10% sucrose was used as marker in each gel. Gels were run at 200 V constant voltage for 45 min. The gels were stained for esterase activity in 100 ml 0.2 *M* sodium phosphate buffer (pH 7.0), 2% (by volume) 20 m*M* α-naphthyl acetate dissolved in acetone, and 0.04 g Fast Garnet GBC Salt (8).

Partial purification of nonspecific esterase. Because it was not possible to maintain greenbug colonies large enough to pro-

vide fresh starting material for purification, greenbugs (adults and nymphs) were collected from sorghum plants and immediately frozen at −80°C. We have routinely stored greenbugs at −80°C for up to 3 months without significant loss of activity (BDS unpublished data). Approximately 1–2 g of frozen aphids were homogenized in Mops [3-(*N*-Morpholino) propane–sulfonic acid] buffer (0.15 *M*, pH 8.0) and the homogenate was centrifuged at 10,000*g* for 20 min. The supernatant was filtered through glass wool and centrifuged at 100,000*g* for 1 hr. The resulting supernatant was applied to a Q-Sepharose Fast Flow ion-exchange column (1.5 × 10 cm) equilibrated with homogenization buffer. The enzyme was eluted with a 10-bed vol salt gradient (0–1.5 *M* NaCl in Mops buffer), and elution profiles were determined for esterase activity and protein. The esterase activity was eluted in one peak, and the fractions containing activity were combined and concentrated in a Spectrum (Houston, TX) stirred cell with a 30,000 MW ultrafiltration membrane. The concentrated fraction was then desalted with a Bio-Rad (Palo Alto, CA) Econo-Pac P-6 desalting column.

A portion of the desalted and concentrated fraction of both resistant and susceptible strains was then applied to a continuous native polyacrylamide electrophoresis gel in a Bio-Rad (Hercules, CA) Mini-Protean II electrophoresis cell. Gels (7.5% polyacrylamide, 1 mm thick) and running buffers contained 0.5 *M* Tris–glycine (pH 8.3) and were run at 200 V for 30 min. Gels were run without sample wells so that a continuous band across the gel was obtained. A 1-cm portion of the gel was removed and stained as described previously (6). After staining was complete, the stained portion was aligned with the remainder of the gel, and the area corresponding to highest esterase activity was cut and homogenized in Mops buffer (0.15 *M*, pH 8.0) with a Teflon glass Potter–Elvehjem homogenizer. The homogenate was centrifuged at 10,000*g* for 10 min to precipitate polyacrylamide gel fragments. Although recovery of activity was low (between 5 and 10%), a single esterase isozyme was observed in native gels of the purified sample stained for activity (data not shown).

Kinetic analysis. Kinetic properties of the semipurified preparations from resistant and susceptible strains were studied by measuring activity toward a series of concentrations of α-naphthyl butyrate. Lineweaver–Burk plots (9) were obtained for four different preparations, and V_{max} and K_m values were calculated for each strain. The mean values of V_{max} and K_m for the resistant and susceptible strains were compared by Student's *t* test.

Inhibition. Each enzyme preparation was assayed for activity, and enzyme concentration was adjusted so that the resistant and susceptible preparations were equal in activity. The preparations were then incubated with varying concentrations of paraoxon in sodium phosphate buffer (0.02 *M*, pH 7.0) in a total of 2 ml solution. The enzyme preparation was assayed for esterase activity toward *p*-nitrophenyl acetate 2 min after addition of the inhibitor. Each reaction mixture (1 ml in total) contained 50 μl inhibited enzyme in 0.02 *M* sodium phosphate buffer (pH 7.0) and 0.5 m*M* *p*-nitrophenyl acetate in acetone. The reaction was started by the addition of *p*-nitrophenyl acetate. Absorbance at 400 nm was read every 6 sec for 2 min and the slope was calculated from the change in absorbance. Slopes and standard errors of inhibition curves were calculated by linear regression and I_{50}'s were calculated for each strain. Each inhibition curve was derived for three different preparations with three determinations for each time point.

RESULTS AND DISCUSSION

Partial purification of a greenbug esterase associated with resistance to parathion was accomplished by the combined techniques

of ion-exchange chromatography and preparative polyacrylamide gel electrophoresis. The initial ion-exchange step resulted in approximately threefold purification with 50–60% recovery. The elution profiles obtained for the resistant and susceptible strains (Fig. 1) indicated nearly identical chromatographic behavior for both protein and activity. The enzyme eluted in a single peak at the same point in the gradient. Similar profiles were observed for three separate preparations from both resistant and susceptible strains. The major difference is seen in the height and area of the activity peak which was consistently two- to threefold greater in the resistant strain. Analysis of esterase composition by native polyacrylamide electrophoresis revealed that the fractions still contained more than one form of esterase isozyme (Fig. 2) although the isozyme associated with resistance predominated. Additionally, it is apparent that

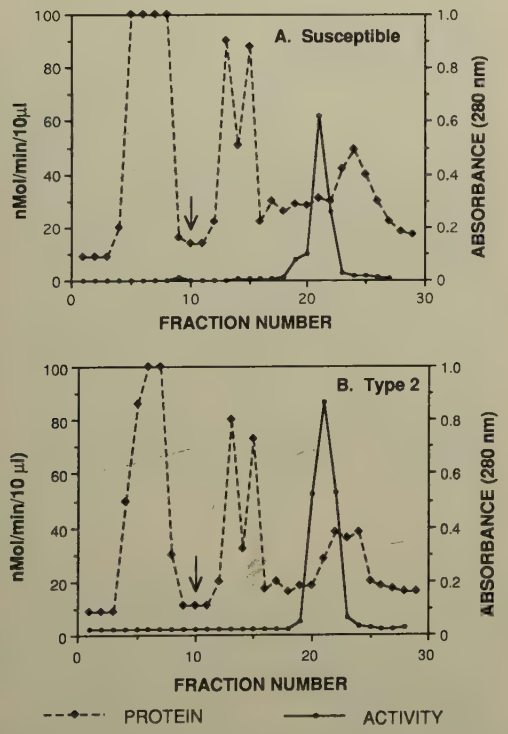

FIG. 1. *Elution profiles for protein and esterase activity using α-naphthyl butyrate as substrate and a Q-Sepharose Fast Flow ion-exchange column. See text for details of chromatography.*

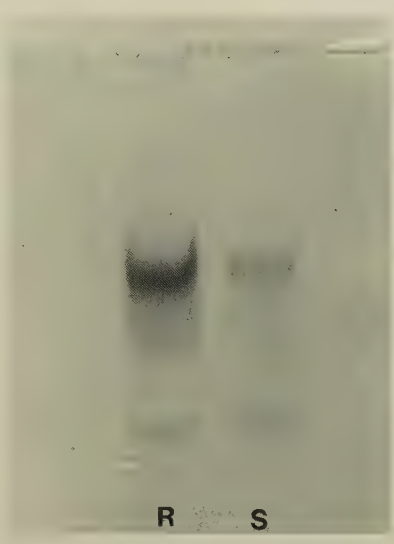

FIG. 2. *Native PAGE gel stained for general esterase activity of fractions obtained after ion-exchange chromatography of susceptible (S) and resistant (R) strains.*

both resistant and susceptible strains exhibited a similar pattern of isozymes except for the single band that exhibited higher staining intensity in the resistant strain.

Because of the presence of more than one esterase isozyme, fractions obtained after ion-exchange chromatography were subjected to preparative native electrophoresis to isolate individual isozymes. Although this technique resulted in poor recovery of activity, it provided a means to obtain individual isozymes that could then be subjected to kinetic and inhibition analyses. Results from kinetic comparisons of preparations purified by native polyacrylamide electrophoresis indicated that K_m values were similar for the two strains although V_{max} was consistently two- to threefold higher in the resistant strain (Fig. 3). Statistical comparisons of K_m values for six different preparations indicated that there was no significant difference in K_m values for the resistant and susceptible strains (Table 1). Identical K_m values but higher V_{max} associated with the resistant strain suggest that the enzymes from the resistant and susceptible strains interact with the substrate identically, and that preparations

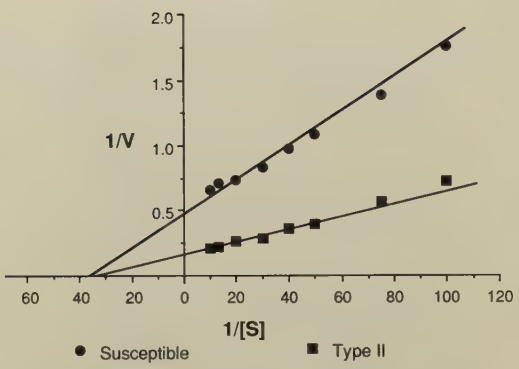

FIG. 3. *Lineweaver–Burk plot of general esterase activity from resistant and susceptible strains obtained after ion-exchange chromatography and preparative electrophoresis.*

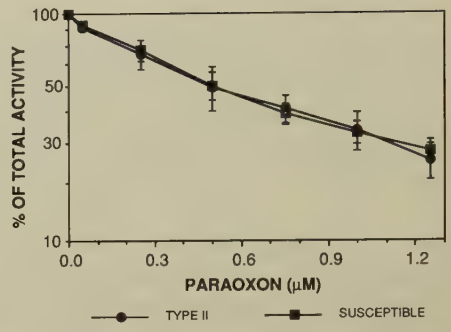

FIG. 4. *Paraoxon inhibition of general esterase activity in fractions obtained from resistant and susceptible strains after ion-exchange chromatography and preparative electrophoresis.*

from the resistant strain may simply have higher titers of enzyme. If preparations from the two strains possessed different enzymes with different catalytic properties, then we would expect to see a difference in K_m values. Conclusive evidence will require purification of the enzyme from both strains to homogenity and determination of turnover numbers.

Results from inhibition experiments using paraoxon as an inhibitor revealed nearly identical inhibition curves with similar I_{50} values for the resistant and susceptible strains (Fig. 4 and Table 1). These results provide further support for the inhibitory nature of paraoxon and the role of inhibition rather than true catalytic hydrolysis as the resistance mechanism (10). If the resistance involved formation of a novel protein with different binding characteristics not present in the susceptible strain, then such similarities in inhibition curves would be unlikely.

The results of this investigation provide evidence that the resistance in the Type II strain of greenbugs likely results from the overproduction of an isozyme that is present in the susceptible strain but at reduced levels. If resistance was the result of changes in DNA sequence resulting in an altered protein that is qualitatively different from the susceptible strain, then differences in properties of the enzyme would have been observed. This was not the case however, as similarities in chromatographic behavior, kinetic properties, and inhibition by paraoxon all indicate the presence of the same isozyme in the resistant and susceptible strain. It is still not clear, however, whether the genetic basis of resistance is the result of multiple copies of the same gene (gene amplification) as has been reported for *Myzus persicae* (11) and *Culex pipiens* (12) or whether overproduction is related to a change in the sequence of regulatory DNA outside the protein coding region resulting in increased protein synthesis.

TABLE 1

Summary of Kinetics and Inhibition of Greenbug General Esterase Associated with Parathion Resistance

Strain	V_{max} (μmol/min/ml) ± SEM[a]	K_m (m*M*) ± SEM[a]	I_{50} ± SEM × 10^{-5} (*M*)[b]
Susceptible	1.49 ± 0.32	0.0206 ± 0.0034	1.74 ± 0.02
Resistant	4.96 ± 1.59[c]	0.0281 ± 0.0088	1.72 ± 0.04

[a] Calculated from the means of four different preparations.
[b] Calculated from the means of three different preparations.
[c] Significantly different from susceptible strain (two-sample *t* test; $P < 0.05$).

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Uptake and Translocation of [^{14}C]MSMA in Cotton and MSMA-Resistant and -Susceptible Cocklebur[1]

RENEE J. KEESE AND N. DWIGHT CAMPER

Department of Plant Pathology and Physiology, Clemson University, Box 340377, Clemson, South Carolina 29634-0377

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Common cocklebur (*Xanthium strumarium* L.) is a competitive weed which interferes with cotton (*Gossypium hirsutum* L.) production throughout the southeastern United States. In South Carolina, an organic arsenical-resistant cocklebur biotype has been identified. Absorption and translocation studies were conducted comparing monosodium methanearsonate (MSMA)-resistant (R) and -susceptible (S)-cocklebur with tolerant cotton using [^{14}C]MSMA. Radioactivity was distributed throughout all plants and average recovery from plants after 1, 3, and 5 days was 98, 70, and 85%, respectively. Greatest concentrations of ^{14}C activity were located in the treated leaf, with both acropetal and basipetal translocation occurring. There were no significant differences in radioactivity recovered from the three plant types. Cocklebur absorbed more MSMA than did cotton; most ^{14}C activity was removed from the treated cotton leaf in the first water rinse. Cotton cuticle appears to play a role in the MSMA tolerance mechanism. No significant ^{14}C root exudations or $^{14}CO_2$ evolution from intact plants was detected. © 1994 Academic Press, Inc.

INTRODUCTION

Common cocklebur (*Xanthium strumarium* L.) is a competitive weed in cotton (*Gossypium hirsutum* L.) throughout the southeastern United States, causing crop yield reductions of 80% (1). Estimates of cocklebur infestations were 605,000 ha in the U.S. cotton belt and 3,970 ha in South Carolina in 1985 (2). Monosodium methanearsonate (MSMA) and DSMA (organic arsenicals) are used postemergence for control of cocklebur in cotton at rates up to 2.2 kg ai ha^{-1} (3). These water-soluble formulations are applied with surfactant to improve leaf wetting and entry (4).

Cocklebur resistance to MSMA was reported in 1988 (5). The susceptible genotypes were controlled in the field with rates of 0.6 kg ai ha^{-1}, while resistant types required 4.5 kg ai ha^{-1} for control. The resistant biotype was not cross-resistant to other herbicides such as atrazine, glyphosate, imazaquin, or paraquat (5). Greater resistance was documented in field versus greenhouse studies and larger plants were more resistant than those less than 1 cm in height (5). Since this first report, other counties in South Carolina (6) as well as other states in the cotton belt have reported organoarsenical resistance in cocklebur (7).

Little is known about the mode of action of the arsenical herbicides, increasing the difficulties in understanding the resistance mechanism. Arsenic toxicity in plants has been documented as a four-stage process: (1) adsorption of arsenic onto plant surfaces, (2) movement of arsenic from the exterior to the interior of roots or tops, (3) translocation to the site of action, and (4) a toxic biochemical reaction (8). Treatment of purple nutsedge (*Cyperus rotundus* L.) with [^{14}C]DSMA caused leaf chlorosis after 3 to 5 days; radioactivity was detected along the tuber chain after 10 to 12 days (9).

[1] Technical Contribution 3292 from SC Agricultural Experimental Station.

[2] Abbreviations used: MSMA, monosodium methanearsonate; DSMA, disodium methanearsonate; R-cocklebur, MSMA-resistant cocklebur; S-cocklebur, MSMA-susceptible cocklebur; dpm, disintegrations per minute; LSS, liquid scintillation spectroscopy; PPFD, photosynthetic photon flux density; RH, relative humidity.

[3] Via Fermenta Plant Protection Company.

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Arsenic and ^{14}C activity were found in the same tissue, demonstrating stability of the carbon–arsenic bond. When johnsongrass (*Sorghum halepense* (L.) Pers.) was treated with [^{14}C]MAA, ^{14}C activity was found throughout treated plants after 4 hr; movement of organoarsenicals occurred rapidly in gramineous species (10).

A methodical set of questions must be answered to describe a resistance mechanism. This research addresses the potential resistance/tolerance mechanism based on differential herbicide absorption and translocation in resistant (R) and susceptible (S) biotypes of common cocklebur. Differential penetration and translocation may be factors both in the tolerance of cotton to MSMA compared to cocklebur and in the resistance mechanism. The objectives of this study were to conduct comparison absorption and translocation studies with [^{14}C]MSMA on the organic arsenical-resistant and -susceptible cocklebur and cotton.

MATERIALS AND METHODS

Plant Material

MSMA-resistant cocklebur burs were originally collected in Lee County, South Carolina, and susceptible burs were collected from Anderson County, South Carolina (5). Germinated seedlings were transplanted to the field in July 1990 and allowed to produce burs. MSMA R-cocklebur plants were sprayed with MSMA (1 kg ha^{-1}) prior to harvest to confirm their resistance. Mature seeds were removed from the bur by cutting it open with a Dremel mototool. The papery sheath was removed from the seed, and seeds were soaked overnight in water. Seedcoats were removed if not previously separated, and the seeds were germinated in sterile petri plates containing moistened filter paper for 2 days at 27°C with a 16-hr photoperiod. Seedlings were then transferred to all-purpose Garden Magic potting mix and placed in a growth chamber (Sherer–Gillett, Marshall, MI) at 23 ± 2°C with a 16-hr photoperiod. These conditions promoted vegetative growth only. Coker 315 cotton seeds were planted approximately 5 days after the cocklebur to provide plants of similar sizes. Cotton plants were also maintained in the growth chamber. Experimental plants were 3 to 6 weeks old, with three to seven true leaves, and 9 to 15 cm tall from soil level.

Herbicide Treatment

[^{14}C]MSMA (Ricerca[3], sp act 6.48×10^{-8} Bq $mmol^{-1}$) solutions were diluted with water to 13,000 dpm μl^{-1}. [^{14}C]MSMA activity of working solutions was determined by liquid scintillation spectroscopy (LSS) at the time of use. A 5-μl droplet (approximately 65,000 dpm and 0.00165 mg MSMA) was applied to the leaf tip of the first fully expanded, oldest leaf of R-cocklebur, S-cocklebur, and cotton plants. This rate is 10 times the field rate if uniformly applied over the plants. The plants were then placed in a growth chamber for 1, 3, or 5 days.

Uptake and Translocation Studies

Treated plants were placed in an incubator (Lab-line, Melrose Park, IL) kept at 25 ± 2°C with a 16 hr photoperiod at 20 μE m^{-2} sec^{-1} photosynthetic photon flux density (PPFD). Experiments were randomized complete block designs across times, with two to three plants per type and duplicate LSS counts per plant part. At specified periods, the treated leaves were washed with 5 ml distilled deionized water for 2 min and dipped in chloroform for 2 min. Both rinse solutions were analyzed by LSS. Plants were then separated into lower plant (root), upper plant (stem and leaves), and treated leaf. Plant material was extracted by grinding in 4 ml boiling water in a mortar and pestle for 1 min; the mortar was rinsed with 1 ml boiling water and added to the extract. Extracts were centrifuged (International Centrifuge, International Equipment Co., Needham Heights, MA) at 12,000 rpm (300*g*) for 5 min. Duplicate 100-μl aliquots

were added to 5.5 ml Ready Safe Scintillation Cocktail and analyzed by LSS.

Autoradiograms

Whole plants were spread on blotting paper, wrapped with Saran Wrap and aluminum foil, and placed in a plant press for 3 days. The blotting paper and plants were then placed in contact with Kodak XAR film (20 × 25 cm), put in a film cassette, and returned to the plant press for 3 weeks for the cocklebur plants and 5.5 weeks for cotton. X-ray films were developed in GBX developer (Sigma Chemical Co.) as per Kodak instructions. All plant parts were later extracted in boiling water and assayed for radioactivity by LSS.

Root Exudation

Plants of R- and S-cockleburs (5–7 cm with 3 or 4 true leaves), and cotton (4–6 cm with 2 true leaves) were treated with 5 μl [^{14}C]MSMA (59,000 dpm), on the tip of the first fully expanded leaf, and placed with roots submerged in a flask containing 250 ml distilled deionized water. Plants were maintained in a greenhouse with a temperature range of 25–30°C for 5 days. Water volume was measured in each flask and 0.5-ml aliquots were sampled and analyzed by LSS. The treated leaf was harvested, ground, and analyzed as previously stated. A completely random design was used, with three plants per type and duplicate dpm counts for each extraction.

CO_2 Evolution

Young plants of each biotype, approximately 10 cm tall, were transplanted into Magenta GA-7 vessels (Carolina Biological Supply Co., Burlington, NC) with potting mix. Plants were watered and a 5-μl droplet of [^{14}C]MSMA (59,000 dpm) was applied to the first fully expanded leaf, and an untreated control was included in both experiments. A plastic scintillation vial was enclosed in each Magenta box containing 5 ml of 0.1 *N* NaOH to trap released CO_2. Containers were kept in a greenhouse, and the experiment was a randomized complete block design. At 1, 3, and 5 days after treatment the NaOH solution was removed and fresh NaOH was added to the vial. Duplicate 500-μl aliquots of NaOH were analyzed by LSS, with cumulative results reported.

Statistical Analysis

An analysis of variance followed by *t* tests was used to determine the effects of plant type, plant part, and day of ^{14}C recovery data. A minimum of four observations was used for statistical determinations. Significant differences are reported at the 5% confidence level.

RESULTS AND DISCUSSION

Growth Chamber Study

Following a 1-day treatment, ^{14}C distribution was analyzed in all three plant types. An average of 91% of the ^{14}C activity was recovered in the treated leaf across all three plant types (Table 1). Approximately 50% of the ^{14}C remained in the cocklebur leaf extract; little activity was found in the cotton leaf extract. Less ^{14}C activity penetrated the cotton leaf compared to the cockleburs. The lowest total recovery was obtained from the R-cocklebur. In the S-cocklebur, radioactivity was detected primarily in the treated leaf, and a small percentage was distributed in the upper plant. The R-cocklebur and cotton both showed some activity in the lower plant portions (4 and 3%, respectively; Table 1). A small amount of ^{14}C activity was also detected by autoradiography in the roots and lower half of the R-cocklebur and cotton (images not shown). LSD analysis indicated no differences in total recovery among the R- and S-cocklebur and cotton plants. Significant quantities of ^{14}C were detected in the treated leaf when compared with other plant portions.

After a 3-day treatment, 64% of the recovered ^{14}C activity remained associated with the treated leaf of all three plant types

TABLE 1
Distribution of [^{14}C]MSMA in Cocklebur and Cotton 1 Day after 63,000 dpm Treatment under Growth Chamber Conditions

Plant part	dpm (% recovered)		
	R-cocklebur	S-cocklebur	Cotton
Treated leaf	52,556 (84%)a	57,900 (92%)a	60,825 (98%)a
Extract	23,616	35,724	5,171
Water wash	28,766	21,770	55,351
Chloroform	175	405	243
Upper plant	0 b	5,765 (9%)b	0 b
Lower plant	2,750 (4%)b	0 b	1,460 (3%)b
dpm recovered	55,378 (89%)a	63,665 (104%)a	62,285 (100%)a

Note. Means within plant type (columns) followed by the same letter are not significantly different at the 5% level according to ANOVA and LSD *t* tests (LSD = 20,260). Means within plant parts were differentiated by ANOVA and LSD *t* tests (LSD = 23,521).

(Table 2). Cocklebur biotypes had 65% of the recovered ^{14}C activity in the leaf extract, directly associated with the tissues. In cotton only 27% of the recovered activity was in the leaf extract, whereas 73% was in the leaf wash. Penetration into cotton tissues appeared to play a role in MSMA tolerance. Plant roots contained approximately 2% of the total activity applied. Autoradiograms of all three plants showed the treated leaf to be darkly imaged, with activity moving down the petiole (Fig. 1). The upper leaves, stems, and roots were also visible, but much fainter than the treated leaf. Radioactivity present above and below the treated leaf indicated translocation from the site of application. Analysis of variance and LSD indicated no differences in radioactivity of the treated leaf or in total recovery from Days 1 and 3 or between plant types. [^{14}C]MSMA appears to move within xylem and phloem, in both directions from the leaf of application.

Radioactivity recovery after a 5-day [^{14}C]MSMA treatment averaged 85%, with 77% of the ^{14}C remaining associated with the treated leaf tissues (Table 3). The S-cocklebur had the lowest total percentage recovery of the plants extracted; cotton recovery was statistically different from the cocklebur biotypes. Cocklebur plants had little ^{14}C activity in the leaf washes, while

TABLE 2
Distribution of [^{14}C]MSMA in Cocklebur and Cotton Plants after a 3-Day Treatment under Growth Chamber Conditions

Plant part	dpm (% recovered)		
	R-cocklebur	S-cocklebur	Cotton
Treated leaf	32,515 (61%)a	34,416 (65%)a	36,343 (65%)a
Extract	22,837	24,369	6,711
Water wash	8,845	9,734	29,339
Chloroform	833	313	293
Upper plant	1,908 (4%)b	1,821 (3%)b	3,439 (6%)b
Lower plant	940 (1%)b	859 (2%)b	875 (2%)b
dpm applied	53,000	53,000	56,000
dpm recovered	35,363 (66%)a	37,096 (71%)a	40,675 (73%)a

Note. Means within plant types (columns) followed by the same letter are not significantly different at the 5% level according to ANOVA and LSD *t* tests (LSD = 12,573). Means within plant parts were differentiated by ANOVA and LSD *t* tests (LSD = 19,301).

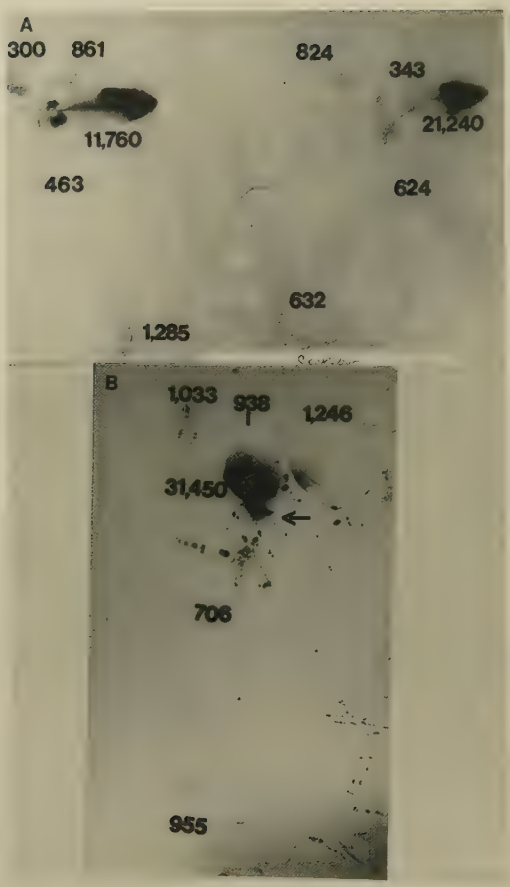

FIG. 1. *Autoradiograms of ^{14}C-treated plants following a 3-day translocation from growth chamber study. (A) R- (left) and S-common cocklebur (right) numbers are dpm's counted by LSS. Both cocklebur plants demonstrate greatest ^{14}C activity in the treated leaves and acropetal and basipetal movement of radioactivity from the point of application. (B) Treated leaf of cotton plant (arrow) is darkly imaged, showing greatest radioactivity dpm counts at the site of application.*

cotton had greater activity in the water fraction. Over time, slightly more radioactivity penetrated into the cotton leaf tissue. The herbicide was washed off in the water fraction, neither penetrating nor binding to the cuticle. Restricted MSMA penetration appears to be responsible for tolerance of cotton to this herbicide, possibly due to the thickness of the cuticle or a specific cuticle constituent. The next largest radioactive fraction was located in the upper plant. Newly formed leaves are imaged on autoradiograms, although not as dark as the treated leaf (Fig. 1). After 5 days, ^{14}C is found throughout the plant, imaging leaves and roots darker than after 3 days. The ANOVA and *t* tests also indicated no significant differences in total recoveries from Days 1 (Table 1, mean = 23,672 dpm), 3 (Table 2, mean = 22,252 dpm), or 5 (Table 3, mean = 23,267 dpm; LSD = 14,977 dpm).

Root Exudation

The presence of ^{14}C activity in roots on autoradiograms led to root exudation experiments. Radioactivity was detected only in hydroponic medium containing R-cocklebur (Table 4). This was insignificant compared to S-cocklebur and cotton, and the ^{14}C activity was minimal (<0.5%) compared to the total applied. The greatest concentration of ^{14}C activity remained in or on the treated leaves of all three plants and more total ^{14}C was recovered from cotton than from the cocklebur biotypes. Root metabolism and exudation of pesticides has been documented in other studies. Root exudations of [^{14}C]MSMA from wheat have been reported; exudation into the soil was followed by $^{14}CO_2$ and [^{14}C]alkylarsine release (11). Other studies have shown root exudation of 2,4-D (>80%) from leafy spurge (*Euphorbia esula*) (12) and from roots of Canada thistle (*Cirsium arvense*) (13). In the Canada thistle study, 2,4-D exudation was greater than that of clopyralid. Root metabolism of picloram and 2,4-D was observed in leafy spurge when the herbicides were coapplied (14). It was concluded from our leaf recovery and exudation results that root exudation was not a factor in the loss of ^{14}C activity.

CO_2 Evolution

The CO_2-trapping experiment attempted to account for losses and locate ^{14}C. If leaf surface chemical reactions were stimulated or microbial degradation was accelerated (15), then $^{14}CO_2$ evolution may be detect-

TABLE 3
Distribution of [^{14}C]MSMA on Cocklebur and Cotton Plants after a 5-Day 57,000 dpm Treatment under Growth Chamber Conditions

Plant part	dpm (% recovered)		
	R-cocklebur	S-cocklebur	Cotton
Treated leaf	40,279 (71%)*a*	40,858 (72%)a	49,596 (87%)a
Extract	20,543	26,708	7,341
Water wash	18,971	13,380	41,809
Chloroform	765	771	446
Upper plant	3,606 (6%)b	2,782 (5%)b	5,058 (9%)b
Lower plant	1,024 (2%)b	776 (1%)b	912 (2%)b
dpm recovered	44,909 (79%)a	44,416 (78%)a	55,566 (97%)a
Plant average	21,747 A	21,745 A	26,310 B

Note. Means within plant type (columns) followed by the same lower case letter are not significant at the 5% level according to LSD *t* tests (LSD = 5,311). Plant means followed by the same upper case letter are not significant at the 5% level according to ANOVA and LSD *t* tests (LSD = 3,552). Means within plant parts were differentiated by ANOVA and LSD *t* tests (LSD = 5,317).

able. Domir *et al.* cited $^{14}CO_2$ evolution from MSMA treatments on wheat, and $^{14}CO_2$ liberation from Canada thistle treated with 2,4-D was proposed (11).

Radioactivity in the NaOH solution was low; under greenhouse conditions (Table 5) the three plant types evolved the same total $^{14}CO_2$. $^{14}CO_2$ evolution increased over time, and Day 1 was significantly less than Days 3 and 5. Only 2.3–7.5% of applied ^{14}C activity was recovered as $^{14}CO_2$. A reduction in radioactivity recovery was not accounted for by $^{14}CO_2$ evolution.

Differential uptake and translocation does not appear to be the mechanism of MSMA resistance in these cocklebur plants, although [^{14}C]MSMA penetration into cotton leaves is reduced, possibly contributing to its MSMA tolerance. Assuming no MSMA degradation, and that the carbon–arsenic bond remains intact, radioactive MSMA was traced throughout the R- and S-cocklebur plants, moving in both directions from the point of application. Unlike MSMA, paraquat resistance in hairy fleabane (*Conyza bonariensis* L.) is based on differential absorption and translocation (16). Cuticular penetration was not affected by paraquat, but herbicide movement was restricted in resistant biotypes by exclusion from the chloroplast. It is not unusual for differential absorption or translocation to be the first potential resistance/tolerance

TABLE 4
Average Radioactivity (dpm) Exuded from Roots of Treated Plants

Sample	Exudate dpm	Treated leaf dpm	Total dpm recovered
R-cocklebur	190 a	32,651 b	32,400 b
S-cocklebur	0 a	32,164 b	32,164 b
Cotton	0 a	38,926 c	38,926 c
LSD (0.05)	—	LSD = 5,339	—

Note. Means followed by the same letter are not significantly different according to LSD tests, $P = 0.05$.

TABLE 5
$^{14}CO_2$ Evolved from MSMA-Treated Plants Grown in a Greenhouse

Treatments	Radioactivity (dpm)			
	Day 1	Day 3	Day 5	Total
Control	0	0	0	0
R-cocklebur	479	122	970	3571 A
S-cocklebur	774	1668	1693	4135 B
Cotton	574	1578	2062	4214 C

Note. Data are presented as average dpm detected from three replicates per treatment, experiment repeated twice. Means for total recovery followed by the same letter are not significantly different, based on LSD *t* test (mean LSD = 1593).

mechanism eliminated. Translocation differences were observed in resistant and susceptible *Hordeum glaucum* in response to paraquat applications (17); in this case the resistance was not due to a reduction in cuticular penetration. Translocation studies on cocklebur with chlorimuron and acifluorfen, commonly used as tank mixes in soybean production, showed that both ^{14}C herbicides translocated from the point of application (18). Similar to results reported here, after 2 days 55% of ^{14}C activity was above that of the treated leaf; acifluorfen was found to reduce absorption and translocation of chlorimuron in cocklebur (19).

Cocklebur showed similar translocation patterns when treated with imazaquin, and leaf penetration was not inhibited with increasing plant maturity (19). When corn plants exhibiting differential tolerance to imazaquin were examined for uptake and translocation differences, none were found (20). Likewise, isoxaben uptake and translocation were not different in resistant strains of *Arabidopsis* (21). Differential glyphosate tolerance in *Lotus corniculatus* L. was not due to the differences in spray retention or translocation (22).

In this study, the greatest concentration of radioactivity remained in the treated leaf, indicating the possibility for a binding reaction in leaf cells/organelles or herbicide sequestration. Radiolabeled MSMA was transported from the application site in both the cockleburs and cotton, as evidenced by the autoradiograms and leaf assays. Minor discrepancies in ^{14}C recovery remain unaccounted for. [^{14}C]Alkylarsine evolution was not determined in the present study in conjunction with $^{14}CO_2$ and may be a metabolic or degradative product (16). Future research will evaluate the site of ^{14}C accumulation and assess membrane integrity and MSMA degradation in the cocklebur biotypes and cotton.

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A Putative Relationship between Hyperglycemic Hormone Degradation and Stress-Induced Protease Activity in the Hemolymph of the American Cockroach

SHIGUI LIU AND JOHN E. STEELE[1]

Department of Zoology, The University of Western Ontario, London, Ontario, Canada N6A 5B7

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Hyperglycemic hormone is released into the hemolymph of cockroaches (*Periplaneta americana*) that have been stressed by immobilization, starvation, or treatment with lindane. The release of hyperglycemic hormone is accompanied by a strong increase in the proteolytic activity of the hemolymph. This appears to be responsible for the rapid destruction of the hyperglycemic hormone both *in vitro* and *in vivo*. The release of protease into the hemolymph of the lindane-poisoned cockroaches is preceded by a lag period. The data show that the proteolytic agent responsible for the destruction of the hyperglycemic hormone is also present in the hemolymph of normal insects but at very low levels. © 1994 Academic Press, Inc.

INTRODUCTION

More than three decades ago Beament drew attention to the similarity of the response by cockroaches to stress resulting from immobilization, enforced sustained activity, and poisoning with DDT (1). Subsequent studies revealed that the hyperglycemic hormones (HGH) are released from the corpora cardiaca (CC) into the hemolymph by treatment with insecticides (2). The release of these hormones *in vivo* as a result of stress thus provides an opportunity to study the mechanisms responsible for the clearance of hormone from the hemolymph under more physiological conditions. The evidence available suggests that proteases may be important in this regard. Neuropeptides, including proctolin (Arg-Tyr-Leu-Pro-Thr), the two adipokinetic hormones, AKHI (pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH2) and AKHII (pGlu-Leu-Asn-Phe-Ser-Thr-Gly-Trp-NH2), and the two myoactive peptides, MI (pGlu-Val-Asn-Phe-Ser-Pro-Asn-Trp-NH2) and MII (pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-NH2) have been shown to undergo proteolytic degradation in the hemolymph, nervous tissue, and Malpighian tubules (3–11) of various insects.

In this study, we have investigated the effect of starvation, immobilization, and lindane treatment on the release of the hyperglycemic hormones in the American cockroach *Periplaneta americana* and followed their subsequent destruction in the hemolymph. The results show that these forms of stress not only induce release of the hyperglycemic hormones but also lead to an increase in the protease activity of the hemolymph which is thought to be responsible for the inactivation of the hormones.

MATERIALS AND METHODS

Experimental Animals

The cockroaches, *P. americana*, used in these studies were obtained from a colony maintained in the laboratory. Only adult males, approximately 1 month old, were used.

Induction of Stress

Chemical stress was imposed by applying 10 μg of lindane (1,2,3,4,5,6-hexachlorocyclohexane, γ-isomer; 1 μg/μl in acetone) to the abdominal tergites. The control insects were treated with 10 μl of acetone.

[1] To whom correspondence should be addressed.

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Physical stress was induced by immobilizing the cockroaches for 6 days in the manner described by Beament (1). To prevent death by dessication the cockroaches were placed in closed containers in which the relative humidity was maintained near saturation.

For some of the experiments the cockroaches were starved for 6 days by holding them in a large jar containing drinking water but no food.

Trehalose Determination

Trehalose was determined using 4 μl of hemolymph which was deproteinized by the addition of 1 ml of 5% trichloroacetic acid (TCA). Trehalose was estimated following addition of 2.5 ml of anthrone reagent to 0.5 ml of the protein-free supernatant (12).

Estimation of Hyperglycemic Hormone Activity in Hemolymph

Hemolymph was obtained for measurement of the HGH activity by centrifugation of the cockroaches in conical centrifuge tubes fitted with a perforated plastic disk to retain the cockroach in the upper end of the tube. The hemolymph was removed at 750 rpm in a refrigerated centrifuge (13) and processed to remove proteins and lipids by the method of Folch *et al.* (14). The final aqueous extract was dried on a rotary evaporator and reconstituted with water to give a volume equal to that of the original hemolymph. The activity of HGH in the hemolymph was estimated by injecting a standard volume of hemolymph extract into a test cockroach which had been neck-ligated 12 hr earlier. The hemolymph of the test insect was removed for trehalose measurement 2 hr after injection. HGH activity was expressed as CC equivalents. This value was obtained by interpolation from a dose–response curve obtained by plotting CC concentration against hemolymph trehalose concentration.

Protease Activity

Hemolymph for protease measurements

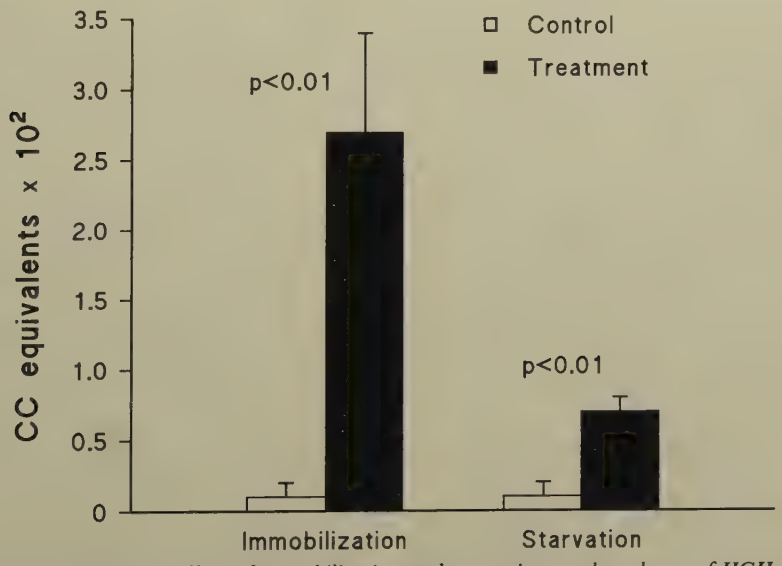

FIG. 1. *The stimulatory effect of immobilization and starvation on the release of HGH into cockroach hemolymph. The hemolymph from both groups of cockroaches was removed on Day 6 after beginning the treatment. Procedures for the estimation of HGH activity in the hemolymph, expressed as CC equivalents, are described under Materials and Methods. The values shown for control hemolymph were obtained using hemolymph from cockroaches subjected to the minimal possible stress. The vertical lines represent the standard error of the mean* ($n = 8$).

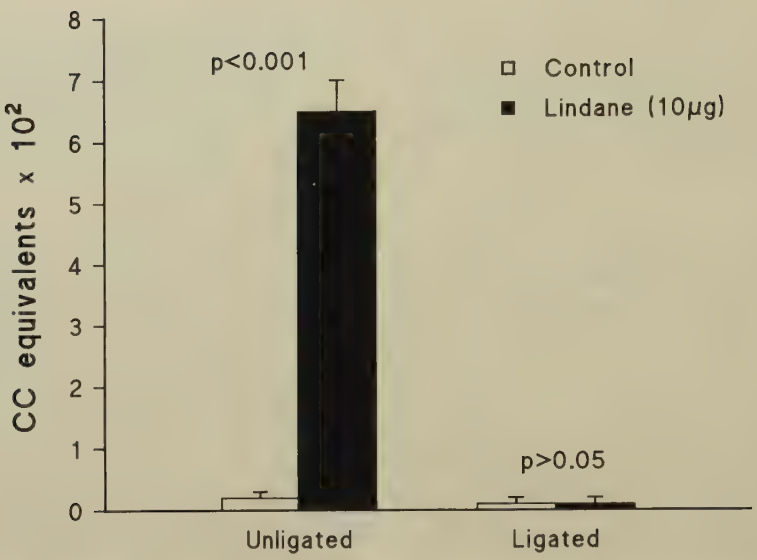

FIG. 2. *The stimulatory effect of lindane on the release of HGH into cockroach hemolymph. The HGH content of hemolymph from lindane-treated and control cockroaches was determined 6 hr after treatment as described under Materials and Methods. The HGH content of the hemolymph is expressed as CC equivalents and was determined as described under Materials and Methods. The vertical lines represent the standard error of the mean (n = 10–12).*

was obtained by centrifugation as described above. The protease activity was determined essentially as described by Baumann (15). Ten microliters of hemolymph was added to 190 μl 0.05 *M* Tris–HCl buffer, pH 8.0, together with 100 μl 1% azocasein dissolved in the same buffer. The mixture was incubated for 1 hr at 30°C. The reaction was stopped by the addition of 1 ml of 10% TCA and the samples were then held at 0°C for 1 hr. After centrifugation at 14,000*g* for 5 min the absorbance of the supernatant was measured at 334 nm. One unit of enzyme activity is indicated by an increase of 0.01 absorbance units per hour. Protein was determined as described by Lowry *et al.* (16).

RESULTS

Figure 1 shows that the low level of HGH activity in the hemolymph of resting cockroaches is increased approximately 25-fold after 6 days immobilization. Starvation for the same period also causes a significant increase in the amount of HGH in the hemolymph but is only 25% as effective as immobilization. Not surprisingly, stress induced by treatment with the insecticide lindane caused more HGH to be released into the hemolymph than did immobilization (Fig. 2; cf. Fig. 1). The amount of HGH present in the hemolymph 6 hr after treatment with the insecticide was approximately double that found after immobilization. The large increase in the concentration of HGH in the hemolymph could be completely blocked by the application of a neck ligature prior to treatment of the insect with lindane (Fig. 2).

Clearance of hormonal regulators from the hemolymph as quickly as possible when interaction with the receptor is no longer required is an essential part of any hormonal mechanism. Figure 3 illustrates an experiment which shows that the hemolymph has the ability to destroy the large amount of HGH released from the corpora cardiaca as a result of stress. Since the HGH activity in the hemolymph is attributable to the presence, either singly or in combination, of two neuropeptides, whose structure previously has been described by

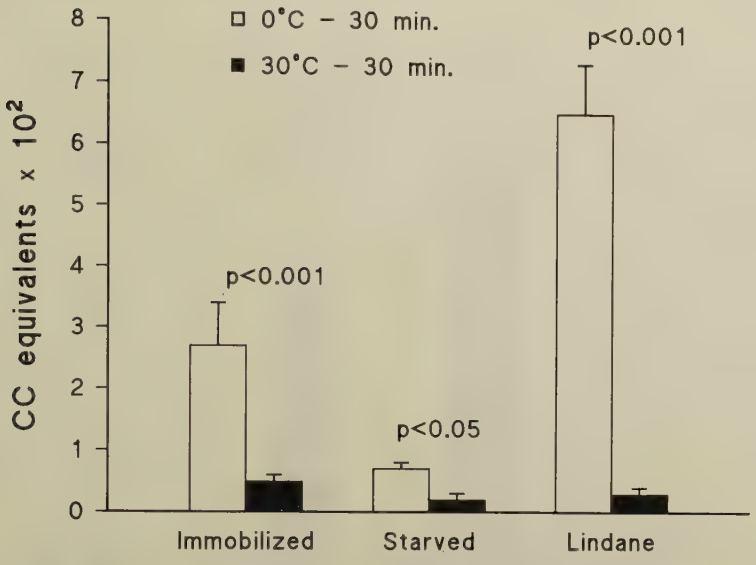

FIG. 3. *Evidence that stress-induced release of HGH is destroyed by proteolytic activity in the hemolymph. The hemolymph obtained following each treatment (immobilization, 6 days; starvation, 6 days; and treatment with 10 μg of lindane, 6 hr) was divided into two aliquots; one was held at 0°C for 30 min and the other incubated at 30°C for 30 min. The HGH was then extracted and assayed as described under Materials and Methods. The vertical lines indicate the standard error of the mean (n = 8–10).*

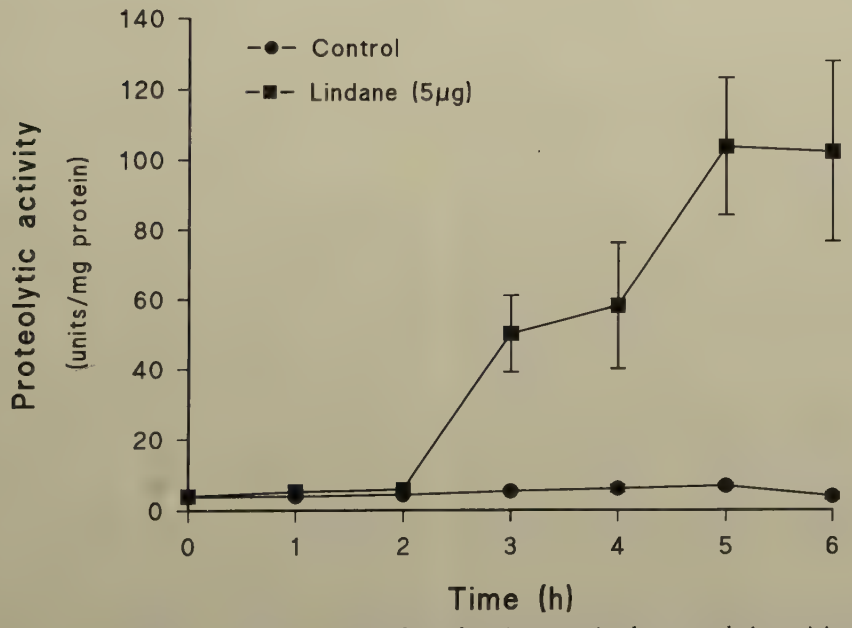

FIG. 4. *An experiment showing the time-dependent increase in the proteolytic activity of hemolymph in lindane-treated cockroaches. In these experiments the insects were treated with 5 μg lindane rather than the customary 10 μg. The method of proteolytic measurement and calculation of the activity is described under Materials and Methods. The values shown are the means ± the standard error of the mean. For lindane-treated cockroaches, n = 12, and for the controls, n = 6.*

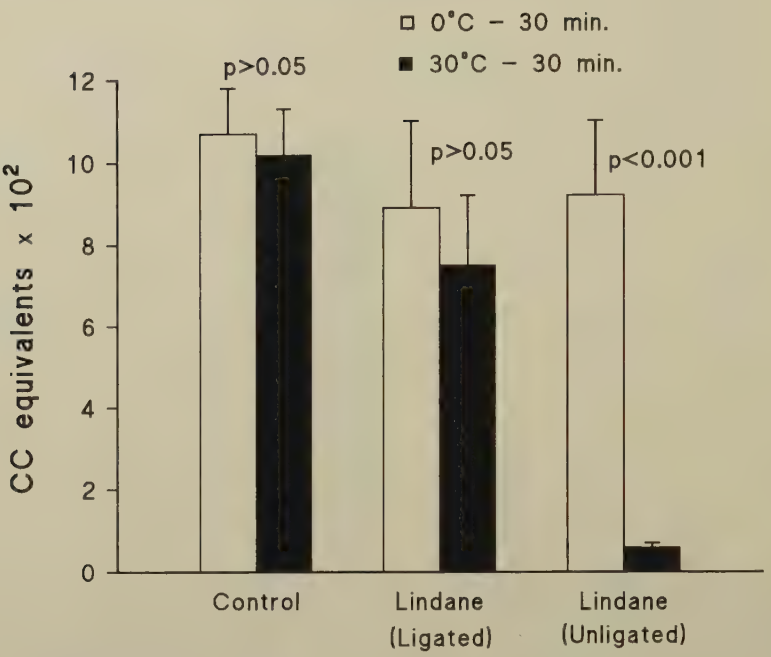

FIG. 5. *Evidence that the appearance of the protease in the hemolymph is dependent on a "head factor." Since neck ligation prevents release of HGH to the abdominal and thoracic hemocoel, a small amount of CC extract as a source of additional HGH was added to the hemolymph obtained from each treatment group. The hemolymph from each group was then divided into two samples; one was held at 0°C for 30 min and the other incubated at 30°C for 30 min before extraction and assay of HGH as described previously. The hemolymph of the untreated cockroaches (control) shows no significant inactivation of the added HGH, whereas that of the lindane-treated (unligated) insects shows considerable inactivation. This destruction of HGH in the hemolymph of lindane-treated cockroaches does not occur if the insects have been neck-ligated prior to treatment with the insecticide. Hemolymph was removed 6 hr after treatment with lindane. The vertical lines represent the standard error of the mean (n = 16).*

Scarborough *et al.* (17), it appears likely that their removal is the result of protease action. The measurement of protease activity in the hemolymph following treatment of the cockroaches with lindane appears to confirm this view. Two hr after treatment with lindane there is a progressive increase in the proteolytic activity of the hemolymph during the following 4 hr (Fig. 4). At 6 hr post-treatment the activity has increased 30-fold over that of the control insects. The view that HGH destruction occurs enzymatically is supported by the observation that HGH activity remains high if the hemolymph samples are held at 0°C.

Evidence that the mechanism for metabolizing the hyperglycemic peptides is controlled by a factor in the head of the insect is shown in Fig. 5. The primary objective in this experiment was to determine whether neck ligation would affect the rate of hyperglycemic peptide removal from the hemolymph. Since ligation prevents the release of hyperglycemic peptides, each sample was fortified with CC extract (1 gland pair) to provide a high level of hormone to serve as substrate for the system responsible for its removal. The data show that the hemolymph of normal cockroaches has no significant effect on the removal of the hyperglycemic peptides even when they are present at a concentration well above the normal level. In contrast, the hemolymph of lindane-treated cockroaches shows a remarkable ability to remove the hyperglycemic hormones from the hemolymph, pre-

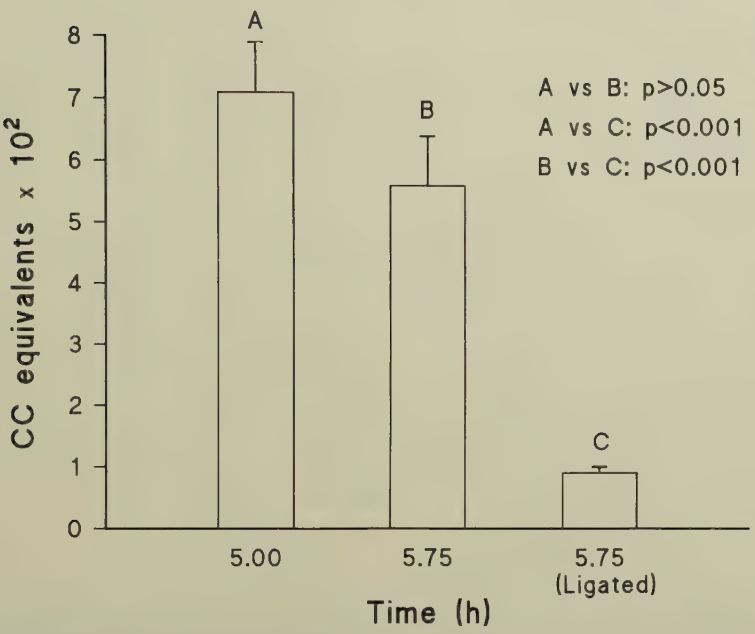

FIG. 6. *An experiment to show that HGH released into the hemolymph by lindane is inactivated in vivo. Each of three groups of 10 cockroaches was treated with lindane (10 μg/cockroach). The hemolymph from the first group was removed 5 hr later for assay of HGH as previously described. Hemolymph from the second group of 10 was removed 5.75 hr after the treatment with lindane. The third group was ligated exactly 5 hr after treatment with lindane and the hemolymph from this group removed for HGH assay 0.75 hr after the application of the ligature. HGH activity is expressed as CC equivalents. The vertical lines indicate the standard error of the mean (n = 8–10).*

sumably by proteolytic action. This ability is lost if the cockroach is neck-ligated prior to the treatment with lindane.

The experiments described above were conducted *in vitro* and thus provide no information about destruction of the hyperglycemic hormones *in vivo*. An experiment that describes the metabolism of the hormones *in vivo* is shown in Fig. 6. In these experiments, lindane stimulated the release of HGH from the corpora cardiaca and promoted production of the factor responsible for its destruction. The application of the ligature 5 hr after the treatment with lindane prevented further release of HGH to the hemocoel, thus permitting destruction of the hormones to manifest itself.

Figure 7 shows that the appearance of the proteolytic factor in the hemolymph is time dependent. No activity was found during the first 2 hr following treatment with lindane. Thereafter, the ability to degrade HGH began to increase steadily, reaching a maximum at 8 hr, after which the activity was held constant for the next 4 hr. These results must be viewed within the context of the actual content of HGH in the hemolymph. Figure 7 (inset) shows that the maximal level of HGH in lindane-treated cockroaches is achieved 6 hr after treatment with the insecticide. During the subsequent 6 hr there is a rapid decline to near basal levels.

None of the experiments described above gave any indication that the HGH inactivation factor may have been present in the hemolymph of normal cockroaches. The possibility that it was absent was suggested by the failure to demonstrate inactivation when the hemolymph was reconstituted to yield its original volume (Fig. 8). To determine whether it may have been present, but because of very low levels causing it to not be easily detectable, the hemolymph extracts were concentrated 10-fold to improve the measurement of HGH

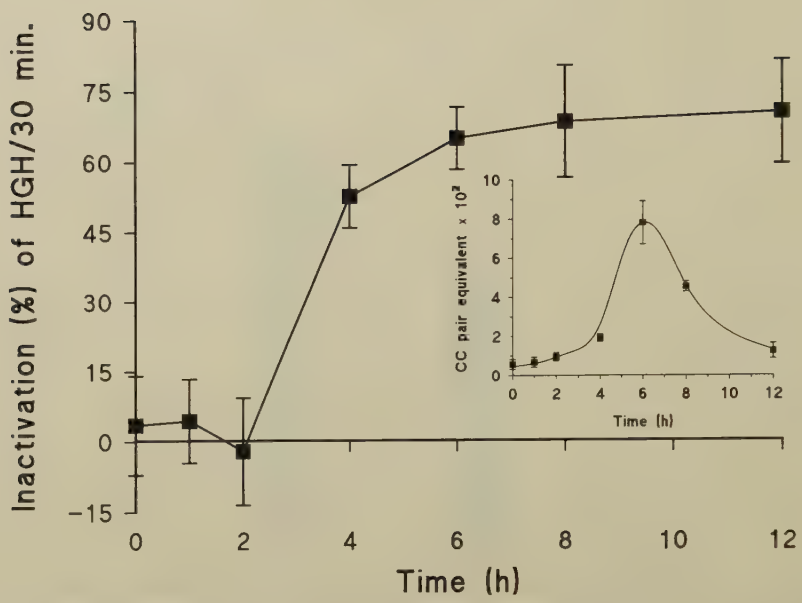

FIG. 7. *The time-dependent inactivation of hyperglycemic activity induced by lindane in cockroach hemolymph. Hemolymph was collected at the times shown from 25 cockroaches which had been treated topically with 10 μg lindane. The hemolymph was divided into two aliquots. One aliquot was incubated at 0°C for 30 min., the other at 30°C for 30 min. After extracting the HGH, its activity was measured by determining its effect on the hemolymph trehalose concentration of test cockroaches. The hyperglycemic activity of each sample following 30 min incubation was obtained by subtracting the hemolymph trehalose concentration obtained for saline-injected control cockroaches from that obtained for the test insects. These values were then converted to CC equivalents by interpolation from the standard curve. The percentage degradation due to incubation of hemolymph at 30°C was calculated as follows: (HGH activity remaining after incubation at 0°C − HGH activity remaining after incubation at 30°C)/HGH activity after incubation at 0°C. The figure inset shows the actual amount of HGH activity present at each time of measurement (n = 8–10).*

activity. The data presented in Fig. 8 show that HGH in resting (untreated) cockroaches can be determined if the hemolymph extracts are reconstituted to yield a concentration which is 10-fold greater than that of the original hemolymph. Under these circumstances, the data show that hemolymph, taken from untreated cockroaches, contains sufficient inactivation factor to destroy the low endogenous level of HGH activity.

DISCUSSION

This study provides the first evidence that a mechanism responsible for the destruction of a circulating neuropeptide in an insect may be regulated by physiological means. This conclusion is based on the finding that severe stress induced by treatment of cockroaches with lindane produces a large increase in the ability of the hemolymph to inactivate the circulating hyperglycemic hormones. The data also show that stress due to immobilization and starvation, which cause less release of hyperglycemic hormone from the CC than treatment with lindane, also leads to a large increase in the activity of the degradative mechanism.

The action of lindane in these experiments has two effects of interest. First, it causes a considerable amount of hyperglycemic hormone to be released from the CC to the hemolymph. This effect is comparable to the release of hyperglycemic hormone to the hemolymph by DDT in the cockroach (2). Second, the release of hyperglycemic hormone is followed by an increase in the activity of the degradative mechanism in the hemolymph. As this

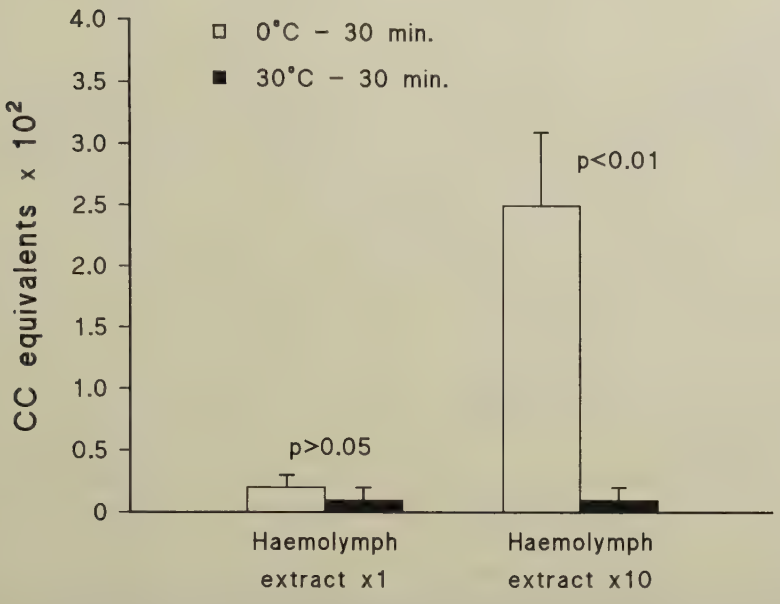

FIG. 8. *An experiment to illustrate the degradation of hyperglycemic hormone in the hemolymph of resting (untreated) cockroaches. Hemolymph from resting cockroaches was collected and divided into two samples. One was incubated at 0°C for 30 min, and the other at 30°C for 30 min. After extraction, the HGH samples were diluted to yield the same volume as that of the original samples of hemolymph. The activity of HGH in the sample was then determined as described under Materials and Methods. The results are shown on the left side of the figure. The hemolymph extracts were then concentrated 10-fold and reassayed. The HGH activity of the concentrated extracts is shown on the right side of the figure. The vertical lines indicate the standard error of the mean (n = 8–10).*

study shows, a large amount of protease makes its appearance in the hemolymph at this time and is presumed to be responsible for the destruction of the hyperglycemic hormone that occurs during the incubation of the hemolymph.

It has been known for many years that cockroaches treated with DDT and malathion display a large increase in the free amino acid content of the hemolymph (18, 19). Although this could be explained by the release of amino acids from the tissues it may have resulted from the action of a protease on hemolymph protein. It is of interest therefore that a strong increase in hemolymph protease has been found in blister beetles, *Mylabrus pustulata,* treated with the insecticide carbaryl (20). The fact that the increase in cockroach hemolymph protease induced by lindane could be blocked by the application of a neck ligature suggests that production of the enzyme may be under the control of a neurohormone. It is also possible that protease production may be promoted by an increase in the titer of hyperglycemic hormone in the hemolymph. This is suggested by the fact that the application of a ligature not only prevents release of the neuropeptides to the hemolymph but also prevents the appearance of the protease. This conclusion is also supported by the fact that the appearance of the proteolytic factor is not related to an effect specific to the action of lindane since the protease also appears to be present in restrained and starved cockroaches.

The source of the proteolytic activity described in this study is unknown. However, because the increase in proteolytic activity is only apparent about 3 hr after treatment with the lindane, this suggests that there is *de novo* synthesis of the enzyme. The protease may be identical to that responsible for the inactivation of proctolin in cock-

roach hemolymph (6, 10, 11) as well as the enzyme shown to inactivate adipokinetic hormone in the hemolymph of *Manduca sexta* (21). Whether it is the same enzyme as that found in the Malpighian tubules of the cockroach (3, 9), which has been shown to inactivate both of the neuropeptides that stimulate trehalose synthesis, remains to be determined.

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NOTICE

The Subject Index for Volume 49 will appear in the November 1994 issue as part of a cumulative index for the year 1994.

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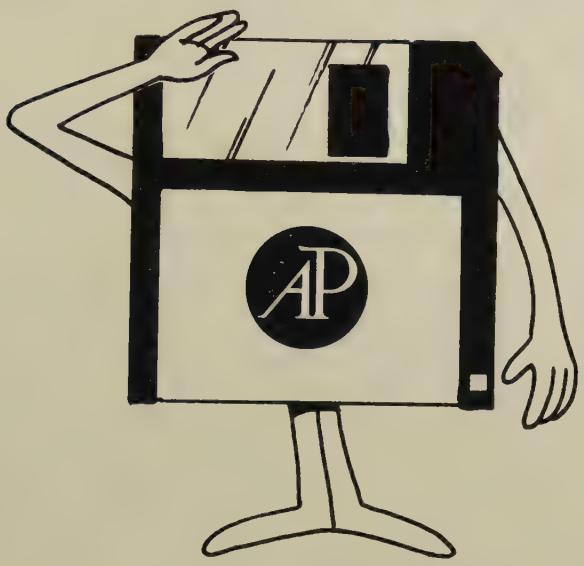

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PESTICIDE BIOCHEMISTRY AND PHYSIOLOGY 49, 155–163 (1994)

Investigation of Effects of Pesticides on Molecular Forms of AChE in Alimentary Canals of Carp

O. KUFCSÁK,* T. SZEGLETES,* G. LÁNG,* K. HALASY,† I. BENEDECZKY,† AND J. NEMCSÓK*

*Department of Biochemistry, A. József University, Közép fasor 52, Szeged, Hungary; and †Department of Zoology, A. József University, Egyetem u. 2, Szeged, Hungary

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All parts of the carp intestine contain three different molecular forms of acetylcholinesterase (AChE): G_1, G_4, and A_{12}. After paraquat treatment there were no significant changes in AChE activity, whereas after $CuSO_4$ treatment there was a slight increase in activity. During exposure of carp to 2 ppm methidathion *in vivo*, the AChE activity in the tissues investigated decreased significantly. Moreover, the relative distribution of the molecular forms of AChE changed relative to that in the control animals. The results suggest that an investigation of the molecular forms of fish AChE could contribute to an understanding of fish AChE at a molecular level and emphasize the importance of *in vivo* and *in vitro* approaches in assessing chemical effects and their potential hazards in the aquatic environment. © 1994 Academic Press, Inc.

INTRODUCTION

From biochemical and pharmacological aspects acetylcholinesterase (AChE) can be ranked among the enzymes that have been intensely studied during the past 20 years (9, 15, 17, 19, 20, 26, 33). Especially important are the results pertaining to the molecular forms of AChE (1, 4–7, 10, 13, 16, 32).

Waste materials originating from intensive agricultural production exert harmful effects when they reach natural waters, since they may become concentrated in the organs of aquatic animals (31). The greater the degree of accumulation, the higher the stage of the given organism in the food chain. Thus, fish are particularly sensitive to environmental contamination of the water and pollutants may significantly damage certain physiological and biochemical processes when they enter the organs of fish (2, 3, 30). In the past 10 years one of our research programs has involved the study of the distribution of the molecular forms of AChE in a phylogenetically lower group of vertebrates and elucidation of the cholinesterase-blocking effects of three pesticides at a molecular level. The pesticides studied were methidathion (MD), an organophosphorus acid ester, paraquat (PQ), an herbicide, and $CuSO_4$, a fungicide.

MATERIALS AND METHODS

Common carp (*Cyprinus carpio* L.) of both sexes, weighing 800–1000 g, were used in the experiments. Three fish were kept in O_2-saturated water at 20 ± 1°C in a 100-liter aquarium. The MD concentration in the aquarium was 2 mg/liter, while that of PQ or $CuSO_4$ was 5 mg/liter. The duration of exposure was 5 days. Each part of the carp intestine (foregut, midgut, and hindgut) was taken from the treated and control fish. One gram of each of the three parts of the alimentary canal was homogenized (10%, w/v) in 12.5 m*M* sodium phosphate buffer (pH 7.2) containing 0.4 *M* NaCl and 0.5% Triton X-100. The suspension was centrifuged (100,000*g* for 2 hr), giving a supernatant and a pellet. AChE was determined in the supernatant, with acetylthiocholine as substrate by the method of Ellman *et al.* (11). Ethopropazine (10^{-5} *M*) was used as a specific inhibitor of BChE. The molecular forms of AChE were separated from 200 μl of supernatant by use of 5–20% (w/v) su-

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crose gradient containing 0.5% (w/v) Triton X-100. The marker enzymes (catalase, EC 1.11.1.6; alkaline phosphatase, EC 3.1.3.1; β-galactosidase, EC 3.2.1.23) were dissolved in 200 μl of the same supernatant. Centrifugation was performed in a VAC 601 Janetzki ultracentrifuge with an 8 * 11 Winkelrotor (100,000*g* for 18 hr at 4°C). Values of $S_{20,w}$ were determined graphically from the curve obtained by plotting the $S_{20,w}$ values of the marker proteins against their positions in the gradient. The following $S_{20,w}$ data were used: β-galactosidase 16.0 S; catalase 11.3 S; and alkaline phosphatase 6.1 S. The relative proportions of each molecular form of AChE were calculated from the ratio of the AChE area in the sedimentation profile to the total area.

A modified version of the method of Koelle and Friedenwald (21) was used for the histochemical detection of possible changes in the AChE activity caused by PQ, $CuSO_4$, or MD. Cleaned gut sections filled with Krebs solution were fixed by immersion in 0.05 *M* cacodylate buffer containing 4% formalin and 2% glutaraldehyde for 6 hr. The preparations were then washed in cacodylate buffer containing an isotonic amount of Na_2SO_4 (19.876 g/liter) for 12 hr, the washing solution being changed in every third hour.

Next, gut sections were incubated at 4 and 25°C for 30 min each. Isotonic Na_2SO_4 incubating mixture (200 cm^3) contained 7 mg of acetylthiocholine iodide, 2.2 cm^3 of 0.1 *M* sodium acetate, 0.1 cm^3 of 3.8% glycine solution, 0.1 cm^3 of 0.1% $Pb(NO_3)_2$ solution, and 0.1 cm^3 of 0.1 *M* $CuSO_4$. The pH was adjusted to 5.5. After incubation, the preparations were washed in isotonic Na_2SO_4 for 3–5 min and then placed in the developing mixture, which contained 20 cm^3 of 1 *N* sodium acetate, 80 cm^3 of distilled water, and 2 g of Na_2S. The pH of the solution was 7.5.

This was followed by washing three times in isotonic Na_2SO_4, then in tap water, and finally in distilled water. The preparations were replaced in the fixing solution for 12 hr after the last washing. Whole-mount preparations were made by cutting rings approximately 1 cm wide from the gut, from which the mucus layer was removed, and the muscular layer was stretched on slides and covered with glycerine under coverslips. Photographs were taken in a Zeiss light microscope.

RESULTS AND DISCUSSION

The gut musculature in the control animals was found to be very rich in AChE-positive nerve elements (Fig. 1). The network of stained nerves extended the entire length and whole thickness of the carp alimentary tract (18). The thickest nerve bundles ran parallel to the longitudinal axis of the gut. Thinner, transversely running nerves connected the bundles at several places. A large number of AChE-positive perikarya were detected between the longitudinal and circular muscle layers, scattered along the nerves. Many were solitary, whereas others gathered in small groups of 2 or 3 cells (Fig. 2). The majority of the neurons were multipolar; the nuclei were free of reaction product.

$CuSO_4$ treatment did not cause histochemically detectable changes in the AChE staining. After PQ treatment, the overall pictures and the pattern of the staining appeared very similar to those for the control, at least at lower magnification. However, at higher magnification a fainter staining, that is, less reaction product in and around the cell bodies, was observed in each gut segment (Fig. 3). MD caused a very obvious decrease in AChE activity compared to the control carp. This agent nearly totally abolished the enzyme activity from both the perikarya and the nerves (Fig. 4).

In the control carp, all the investigated parts of the alimentary tract (stomach, midgut, and hindgut) contained three different molecular forms of AChE: G_1, G_4, A_{12}. Form G_4 occurred in the highest amount, accounting for 51.1–74.1% of the total AChE activity (Fig. 5). Its proportion increased in the anal direction, while that of

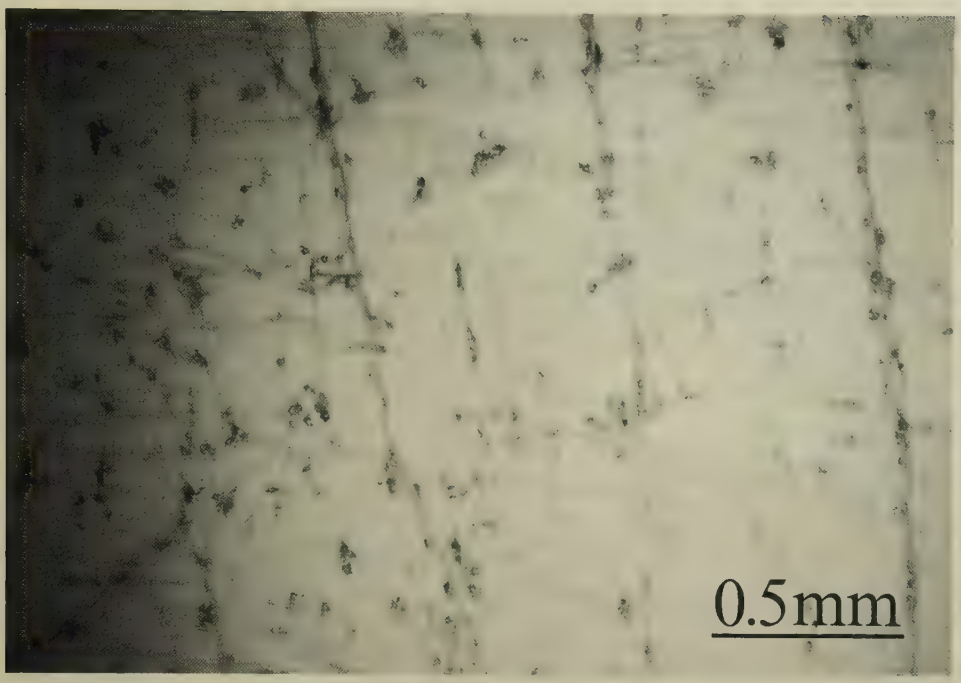

FIG. 1. *Picture of AChE-positive nerve elements of control carp gut musculature at lower magnification. Whole-mount preparation (×50.4).*

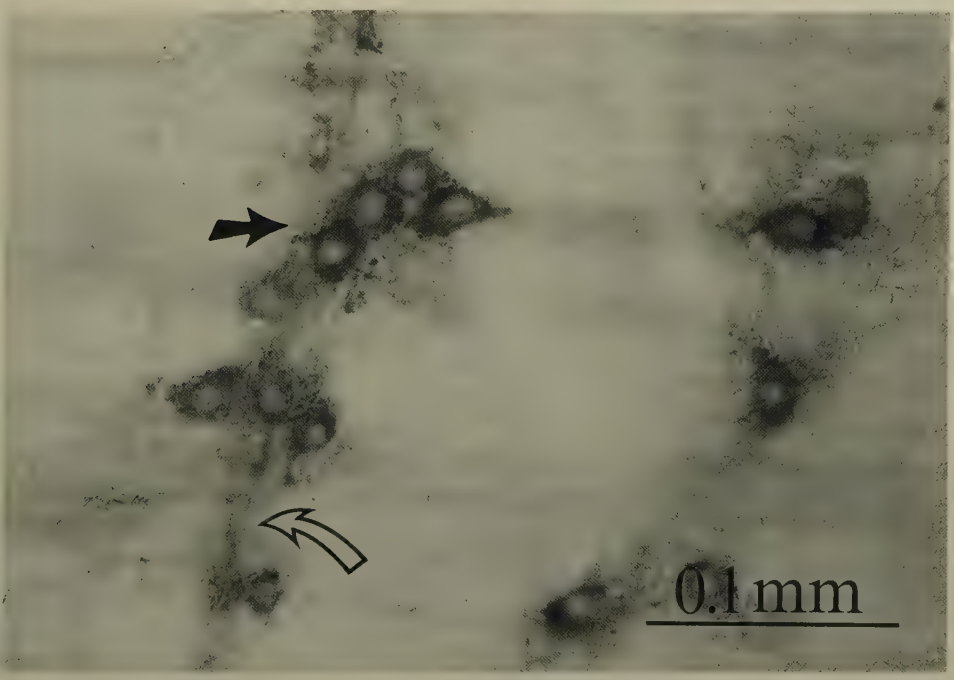

FIG. 2. *A group of strongly AChE-positive cell bodies (solid arrow) along more faintly stained nerve fibers (open arrow) in myenteric plexus of control carp. Whole-mount preparation (×320).*

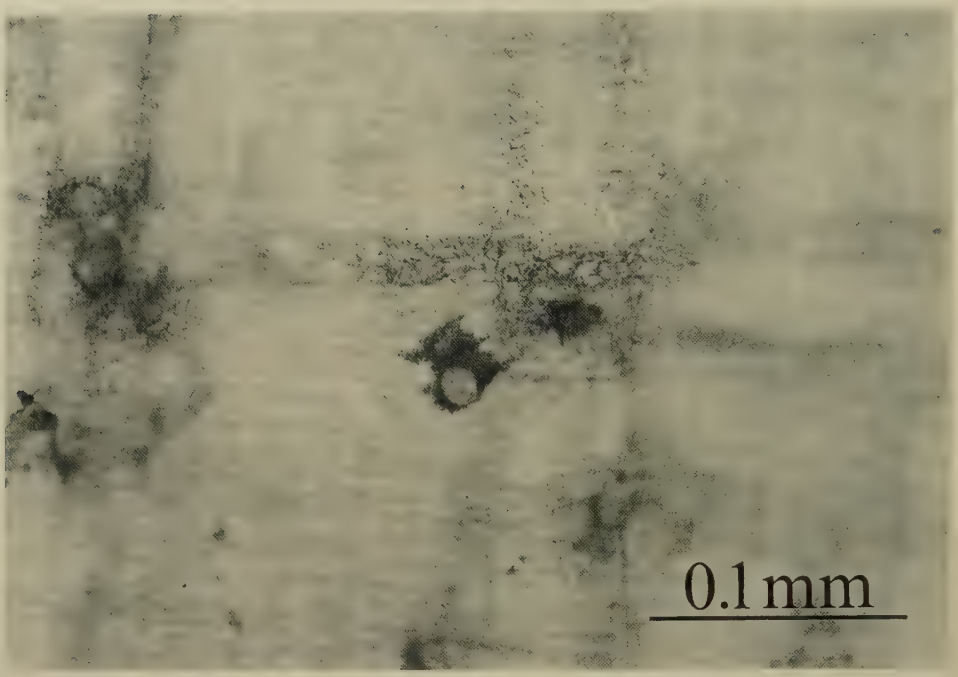

FIG. 3. *AChE-positive elements in PQ-treated carp intestine at higher magnification. Whole-mount preparation* (×*320*).

FIG. 4. *AChE-positive nerve elements in MD-treated carp midgut. After exposure, the reaction products were hardly visible* (*cell body—solid arrow*). *Whole-mount preparation* (×*320*).

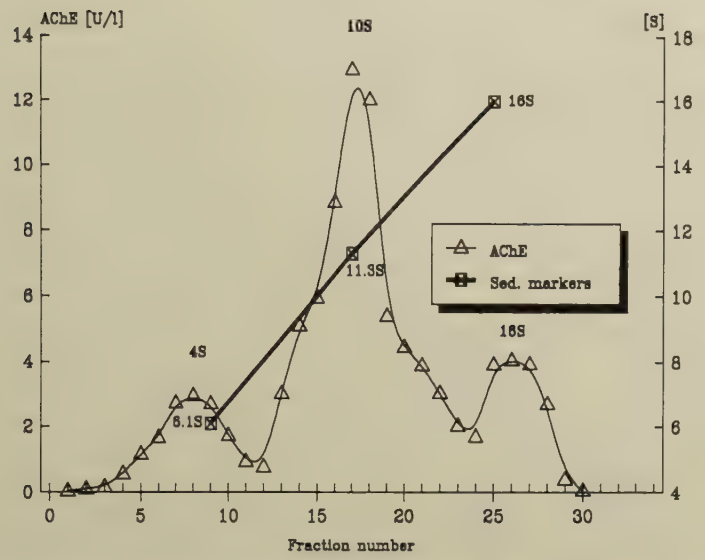

FIG. 5. *Sedimentation velocity profiles of different molecular forms of AChE in control carp midgut. Samples were homogenized in 12.5 mM sodium phosphate buffer, pH 7.2, containing 0.4 M NaCl and 0.5% Triton X-100. After centrifugation at 100,000g for 2 hr, 200 μl of supernatant was mixed with markers, layered on a 5–20% sucrose gradient, and centrifuged at 100,000g with a VAC 601 Janetzki 8 * 11 Winkelrotor for 18 hr at 4°C. All fractions were analyzed for AChE and enzyme markers [catalase (EC 1.11.1.6), 11.3 S; β-galactosidase (EC 3.2.1.23), 16.0 S; and alkaline phosphatase (EC 3.1.3.1), 6.1 S].*

form G_1 decreased (Table 2). The relatively high amount of form A_{12} (13.2–16.9%) in the carp gut was striking. In our earlier experiments, relatively high amounts of form A_{12} were found in carp liver and brain (3.7 and 21.1%, respectively) (24). Form A_{12} is known to be present in decreasing proportions in organisms of phylogenetically higher orders (29). Since fish are a class of vertebrates at one of the lowest phylogenetic levels, this would explain the high A_{12} content relative to that in higher vertebrates (Fig. 5, Table 2).

In response to MD treatment, the amounts of all three molecular forms of AChE significantly decreased ($P < 0.01$ in foregut and midgut AChE activity, $P < 0.05$ in hindgut AChE activity, Student's t test; Fig. 6, Table 1). The distribution of the AChE molecular forms after MD treatment showed significant changes, except the portion of form G_1 in the foregut, which almost remained at the same level ($P < 0.05$ in form G_1 in MD-treated midgut and in form G_4 in foregut, and $P < 0.01$ in all other molecular forms of AChE in MD-treated intestine, which have not been mentioned yet). In our earlier work, the AChE level was inhibited by 80%, even 2 weeks following MD treatment at the same concentration (23). The LC_{50} in ppm in carp at 96 hr was 5.5, and the half-life of MD in water at 20°C was 6 days (unpublished results). The organophosphorus acid esters are known to inhibit AChE activity in fish (9, 14, 25), the reason for this being that these compounds undergo covalent binding with the seryl-OH group of the enzyme. In our experiments, AChE inhibition was evidenced in the alimentary tract of carp at this comparatively high (2 ppm) MD concentration. The reason may be that the AChE found in carp reacts to inhibitors more slowly than that in mammals (22), which may be due to the lower body temperature of fish. In fish living in natural waters, even a relatively low concentration of organophosphorus acid ester may cause considerable AChE

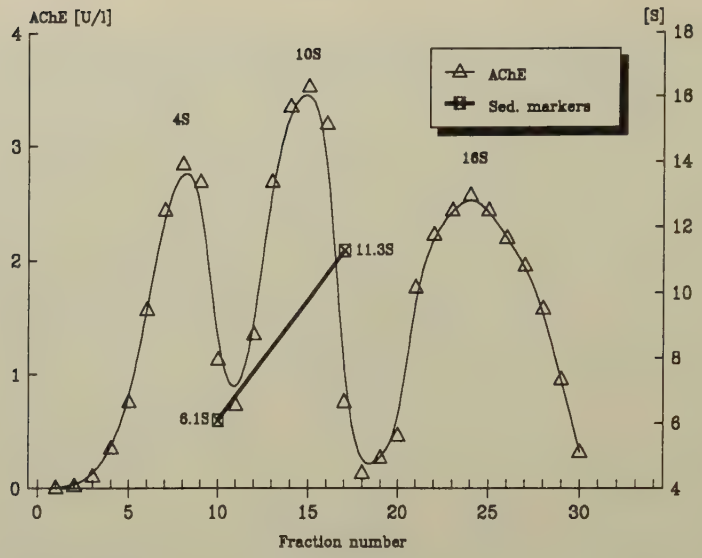

FIG. 6. *Sedimentation velocity profiles of different molecular forms of AChE in MD-treated carp midgut. For experimental conditions, see Fig. 5.*

inhibition (34), which might be a consequence of the intense accumulation of the insecticide in fish (28). We suggest that such a compound does not only block the active center of the enzyme, but also drastically inhibits *de novo* synthesis. In other animal species, for example birds, it has been demonstrated that, following organophosphorus acid ester exposure, the AChE activity was restored only 26 days after 55–64% inhibition, mainly as a result of *de novo* AChE synthesis (12).

The consequences of treatment with sublethal concentrations of organophosphates, such as MD, or a group of related compounds are behavioral effects (for instance, feeding); chronic effects on the central nervous system; mutagenic, carcinogenic, teratogenic, and porphyric effects; and effects on the immune system, on hormones, on the reproductive system, on the retina, and on lipid metabolism. Many are directly or indirectly connected with an AChE inhibition (27, 35).

No significant change in activity was observed following the 5-day PQ treatment (Table 1). The only larger difference detected was in the proportion of form G_1 in foregut and form A_{12} in midgut ($P < 0.05$, Fig. 7, Table 2). Meanwhile, marked elevations of forms G_1, G_4, and A_{12} were measured after $CuSO_4$ treatment ($P < 0.01$, form G_1 in the midgut; $P < 0.05$, form G_1 in hindgut, forms G_4 in foregut and midgut,

TABLE 1

AChE Activities in Intestines of Carp Treated with 5 ppm of PQ, 5 ppm of $CuSO_4$, or 2 ppm of MD, Compared with Those in Control Fish

	AChE activity in homogenates (U/liter)			
	Control	PQ	$CuSO_4$	MD
Foregut	155.4 ± 18.1	149.3 ± 10.1	148.1 ± 21.2	68.9 ± 5.4
Midgut	112.8 ± 9.7	99.2 ± 23.6	137.1 ± 8.0	50.6 ± 9.3
Hindgut	107.8 ± 11.2	109.1 ± 14.6	172.8 ± 23.5	76.3 ± 12.5

Note. Exposure time 120 hr; water temperature 20 ± 1°C. AChE activity is expressed in U/liter homogenate (1 U:1 μmol substrate * min^{-1}).

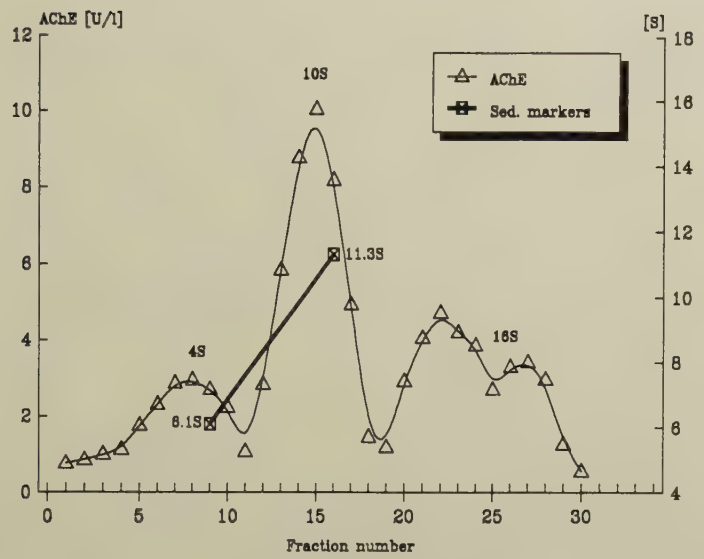

FIG. 7. *Sedimentation velocity profiles of different molecular forms of AChE in PQ-treated carp midgut. For experimental conditions, see Fig. 5.*

and forms A_{12} in midgut and hindgut; Fig. 8; Tables 1 and 2).

The reason may be that PQ and $CuSO_4$ decreased the activity of AChE after short-term exposure—as we measured earlier (23)—but might also enhance the *de novo* synthesis. Regeneration of AChE activity after the 5-day PQ and $CuSO_4$ treatments might depend on the *de novo* synthesis rather than on reactivation of the inhibited AChE. Thus, the elevated AChE activity after the 5-day PQ and $CuSO_4$ treatments might be a consequence of the fact that $CuSO_4$ primarily decreases the already synthesized AChE activity, but stimulates its *de novo* synthesis.

TABLE 2

Distribution of Different Molecular Forms of AChE in Carp Alimentary Canals before and after Treatment with 5 ppm of PQ, 5 ppm of $CuSO_4$, or 2 ppm of MD

Condition	AChE molecular forms (%)		
	G_1	G_4	A_{12}
Control foregut	32.3 ± 5.0	51.1 ± 8.6	16.6 ± 3.1
Control midgut	19.1 ± 3.3	64.0 ± 10.4	16.9 ± 2.1
Control hindgut	12.7 ± 1.7	74.1 ± 9.3	13.2 ± 1.2
PQ-treated foregut	18.8 ± 0.3	64.0 ± 15.6	17.2 ± 0.7
PQ-treated midgut	22.0 ± 2.6	43.7 ± 3.6	34.3 ± 2.8
PQ-treated hindgut	14.6 ± 0.5	69.0 ± 10.1	16.4 ± 3.2
$CuSO_4$-treated foregut	22.9 ± 6.3	64.3 ± 9.6	12.8 ± 0.7
$CuSO_4$-treated midgut	1.3 ± 0.2	89.9 ± 14.6	8.8 ± 1.8
$CuSO_4$-treated hindgut	7.3 ± 1.5	76.2 ± 10.3	16.5 ± 1.2
MD-treated foregut	43.3 ± 6.3	33.4 ± 9.6	23.3 ± 0.7
MD-treated midgut	25.8 ± 2.6	31.0 ± 3.6	43.2 ± 5.8
MD-treated hindgut	35.5 ± 3.5	28.7 ± 6.1	35.8 ± 3.2

Note. Exposure time 120 hr; water temperature 20 ± 1°C. Values are expressed as percentages (±SEM) of total molecular forms of AChE and are the averages of the data on three or four fish.

In conclusion, this paper provides a histo- and biochemical characterization of AChE and its molecular forms in the entire alimentary canal of control and pesticide-treated carp. The results indicate that organophosphorus compounds do not only inhibit AChE activity, but also exhibit changes in the distribution of molecular forms. MD probably inhibits the *de novo* synthesis of AChE. Although $CuSO_4$ and paraquat did not affect this enzyme directly, they can influence its activity by causing an elevated AChE synthesis. Furthermore, our results suggest that fish AChE and its molecular forms might be good biomonitoring tools for the assessment of chemical effects and their potential hazards in the aquatic environment.

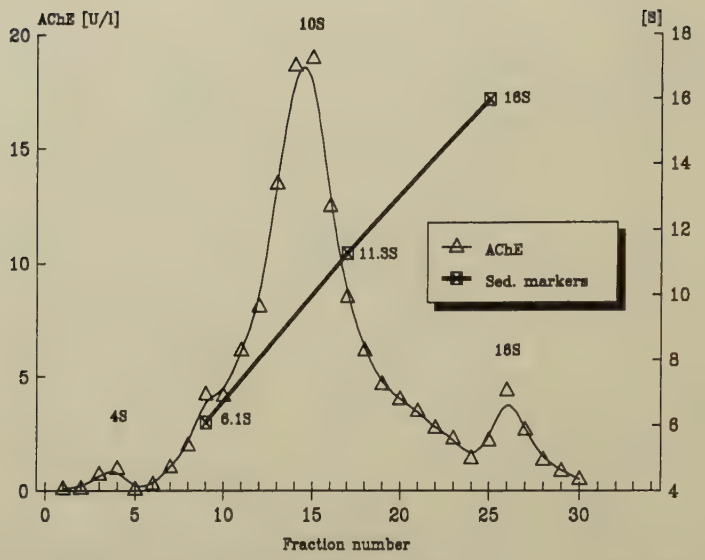

FIG. 8. *Sedimentation velocity profiles of different molecular forms of AChE in $CuSO_4$-treated carp midgut. For experimental conditions, see Fig. 5.*

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Steric Aspects in the Fungicidal Activity of *O,O*-Bisaryl Isopropyl Phosphonates[1]

E. S. J. NIDIRY[2] AND N. K. ROY

Division of Agricultural Chemicals, Indian Agricultural Research Institute, New Delhi-110 012, India

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A comparative study of various steric parameters for *O,O*-bisaryl isopropyl phosphonate fungitoxicants showed that a steric effect of substituents at the *para* position of the phenyl ring is best represented by molar refractivity, which shows that a bulk tolerance problem rather than a directional steric effect is involved. The relationship between fungitoxicity and molar refractivity of the *para* substituent is parabolic in nature. The steric effect of substituents at the *meta* position of the phenyl ring, which played a comparatively minor but statistically significant role in determining fungitoxicity to two fungi, is better represented by Taft's steric parameter or sterimol parameter for maximum width, which shows that this steric effect is directional in nature. The fitting of *O,O*-bisaryl isopropyl phosphonates through the *para* position of the phenyl ring into the cleft of the bioreceptor appears to be the crucial step in the mode of action. © 1994 Academic Press, Inc.

INTRODUCTION

The steric effects of substituents on fungicidal activity have received a great deal of attention (1). The first and still most widely used parameters for steric substituent effects in pesticide design are Taft's E_s constants, which were developed in studies of organic reaction mechanisms of aliphatic esters as correction parameters for intramolecular effects of substituents on a nearby reaction center (2). Unger and Hansch (3) pointed out that since the main mechanism in the biomedicinal system is "receptor fit," in which a substituent must fit into the narrow cleft of the bioreceptor, steric effects will be more or less satisfactorily modeled by molar refractivity (MR). MR is a corrected form of molar volume and a measure of general steric bulk (4, 5). Verloop *et al.* (6) undertook a multiparameter approach in which they selected five dimensions for each substituent and developed a computer program using Van der Waal's radii, standard bond angles and lengths, and reasonable conformations to define the space requirements of a substituent. The five dimensions were designated *L*, *B*1, *B*2, *B*3, and *B*4. Verloop and Tipker discussed the applications of sterimol parameters in the study of fungicidal selectivity (1).

In the QSAR studies on *O,O*-bisaryl isopropyl phosphonate fungitoxicants (7–9), we found that steric effects play a dominant role in determining the fungitoxicity of the series. We present here the detailed comparative study of the steric effects using the parameters E_s, MR, *L*, *B*1, *B*2, and *B*4.

METHODS

Toxicity Data and Biological Materials

$-log\ EC_{50}$, where EC_{50} is the median effective molar concentration for mycelial growth inhibition on potato dextrose agar medium, for the following phytopathogenic fungi were taken as the dependent variables:

(1) *Helminthosporium oryzae*
(2) *Pyricularia oryzae*
(3) *Alternaria alternata*
(4) *Rhizoctonia bataticola*
(5) *Fusarium solani*
(6) *Sclerotium rolfsii*. These data are presented in Table 1.

[1] Contribution 534.

[2] Present address: Indian Institute of Horticultural Research, Hessaraghatta Lake P.O., Bangalore-560 089, India.

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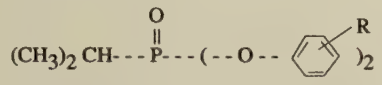
TABLE 1
Fungitoxicity Data of O,O-Bisaryl Isopropyl Phosphonates



Compound	*R*	$-\log EC_{50}$ (mol/liter) against test species					
		H. oryzae	*P. oryzae*	*A. alternata*	*F. solani*	*R. bataticola*	*S. rolfsii*
1	H	3.42	3.30	3.35	2.88	3.48	3.47
2	2-Cl	4.53	3.28	3.17	<2.54[a]	4.21	3.84
3	4-Cl	3.52	2.87	2.84	<2.54[a]	4.09	3.99
4	$2\text{-}OCH_3$	3.41	3.09	3.08	2.93	3.73	2.87
5	$4\text{-}OCH_3$	3.59	3.27	3.17	3.12	3.63	3.59
6	$2\text{-}CH_3$	4.39	3.97	3.46	2.91	3.99	3.26
7	$3\text{-}CH_3$	3.81	3.92	3.66	2.98	3.95	3.54
8	$4\text{-}CH_3$	4.05	3.63	3.40	3.39	3.95	3.69
9	$2\text{-}NO_2$	3.55	3.35	3.13	2.55	3.97	3.38
10	$4\text{-}NO_2$	2.56	2.75	2.02	<2.56[a]	3.76	3.10
11	$4\text{-}C(CH_3)_3$	<2.59[a]	<2.59[a]	<2.59[a]	<2.59[a]	<2.59[a]	<2.59[a]
12	$4\text{-}SCH_3$	2.69	2.14	2.24	<2.57[a]	3.92	2.64
13	$2,3\text{-}(CH_3)_2$	4.18	3.56	3.18	<2.52[a]	4.20	2.84
14	$2,4\text{-}(CH_3)_2$	4.27	3.07	2.90	<2.52[a]	4.16	3.47
15	$2,5\text{-}(CH_3)_2$	4.16	3.45	3.20	<2.52[a]	4.23	3.34
16	$3,4\text{-}(CH_3)_2$	3.88	2.93	2.90	<2.52[a]	4.27	2.89
17	$3,5\text{-}(CH_3)_2$	4.25	3.82	3.34	<2.52[a]	4.37	3.39
18	$2,3,5\text{-}(CH_3)_3$	4.22	3.61	<2.56[a]	<2.56[a]	4.45	2.83
19	$2,4,5\text{-}Cl_3$	4.71	4.76	4.14	4.15	4.58	4.15
20	Cl_5	4.64	4.54	4.15	3.76	4.33	3.83

[a] These values correspond to $EC_{50} > 1$ mg/ml.

Chemical Descriptors

The hydrophobicity parameter $\Sigma\pi$ for each compound was calculated by simple addition of Hansch–Fujita's substituent constants characterizing hydrophobicity (4). Electronic parameter $\Sigma\sigma$ for each compound was calculated by addition of σ_p and σ_m values from literature (4) and presuming $\sigma_p = \sigma_o$. For the *ortho* substituent, σ_p along with the Swain–Lupton constant F' was taken into consideration. The steric parameters E_s, MR, *L*, *B*1, *B*2, and *B*4 were designated according to their position on the phenyl ring. Thus, E_s (*o*) denotes Taft's steric parameter for the *ortho* substituent, $E_s(m)$ that for the *meta* substituent, and $E_s(p)$ that for the *para* substituent. Other steric parameters were also designated in a similar manner (Table 2). The additive nature of all substituent parameters was presumed. The square terms of $\Sigma\pi$, MR(*p*), and all the sterimol parameters also were taken into consideration. The various parameters were considered for only one phenyl ring since the substitution pattern of both rings is exactly the same for all of the compounds.

Multiple Regression Analysis

The stepwise regression given in the SPSSG program of Burrough's electronic computer was used (10). In the first stage of analysis, regression was done by taking only one type of steric parameter for *ortho*, *meta*, and *para* substituents. From the first stage, it was clear that the steric effect of the *para* substituent is best represented by MR(*p*). Thus, MR(*p*) was retained in the

TABLE 2
Descriptor Variables of Substituents

Substituent	Descriptor variables									
	π	σ_m	σ_p	F'	E_s	MR	L	$B1$	$B2$	$B4$
H	0.00	0.00	0.00	0.00	0.00	1.03	2.06	1.00	1.00	1.00
CH_3	0.56	−0.07	−0.17	−0.04	−1.24	5.65	3.00	1.52	1.90	2.04
Cl	0.71	0.37	0.23	0.41	−0.97	6.03	3.52	1.80	1.80	1.80
NO_2	−0.28	0.71	0.78	0.67	−2.52	7.36	3.44	1.70	1.70	2.44
OCH_3	−0.02	0.12	−0.27	−0.26	−0.55	7.87	3.98	1.35	1.90	2.87
SCH_3	0.61	0.15	0.00	0.20	−1.07	13.82	4.30	1.70	1.90	3.26
$C(CH_3)_3$	1.98	−0.10	−0.20	−0.07	−2.78	19.62	4.05	1.90	2.76	3.49

second stage and the parameters of *ortho* and *meta* substituents were changed. The results of the multiple regression analysis are given in the next section along with statistical values.

RESULTS

The results of the multiple regression analysis are discussed for all the test fungi. The various equations are given together with their statistical values: n, R, s, and F. Apart from giving these conventional statistical parameters, we have tested the reliability of equations in a different manner, that is, by seeing whether these equations can explain the very low activity [EC_{50} (mg/ml) > 1 and $-\log EC_{50}$ (mol/liter < 2.59] of *O,O*-bis(4-tertiary butyl phenyl) isopropyl phosphonate which was excluded from the regression analysis (Table 4).

Helminthosporium oryzae

Equations [1] to [6] give a comparison of the various parameters used for the steric effect of a substituent at the *para* position of the phenyl ring.

$$-\log EC_{50} = 3.694 + 0.456\,\Sigma\pi + 0.338\,E_s(p) \quad [1]$$

$$n = 19,\ R = 0.794,\ s = 0.384,\ F = 13.63$$

$$-\log EC_{50} = 3.708 + 0.438\,\Sigma\pi - 0.0069\,[MR(p)]^2 \quad [2]$$

$$n = 19,\ R = 0.859,\ s = 0.323,\ F = 22.49$$

$$-\log EC_{50} = 3.955 + 0.489\,\Sigma\pi - 0.060\,[L(p)]^2 \quad [3]$$

$$n = 19,\ R = 0.823,\ s = 0.358,\ F = 16.88$$

$$-\log EC_{50} = 3.954 + 0.556\,\Sigma\pi - 0.297\,[B1(p)]^2 \quad [4]$$

$$n = 19,\ R = 0.810,\ s = 0.370,\ F = 15.27$$

$$-\log EC_{50} = 4.109 + 0.491\,\Sigma\pi - 0.454\,B2(p) \quad [5]$$

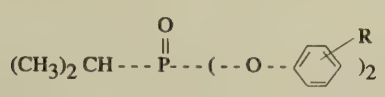
$$n = 19,\ R = 0.759,\ s = 0.413,\ F = 10.73$$

$$-\log EC_{50} = 3.836 + 0.414\,\Sigma\pi - 0.099\,[B4(p)]^2 \quad [6]$$

$$n = 19,\ R = 0.824,\ s = 0.338,\ F = 16.92.$$

The above equations show that molar refractivity represents the steric effect of the *para* substituent better than Taft's steric constant and sterimol parameters. Observed and calculated values of $-\log EC_{50}$ based on Eq. [2] are given in Table 3 for

TABLE 3
Experimental and Calculated Values of Fungitoxicity of O,O-Bisaryl Isopropyl Phosphonates to H. oryzae



Compound	*R*	Exp	Calc[a]
1	H	3.42	3.70
2	2-Cl	4.53	4.01
3	4-Cl	3.52	3.77
4	2-OCH_3	3.41	3.69
5	4-OCH_3	3.59	3.27
6	2-CH_3	4.39	3.95
7	3-CH_3	3.81	3.95
8	4-CH_3	4.05	3.73
9	2-NO_2	3.55	3.58
10	4-NO_2	2.56	3.21
11	4-$C(CH_3)_3$	<2.59	1.92
12	4-SCH_3	2.69	2.66
13	2,3-$(CH_3)_2$	4.18	4.19
14	2,4-$(CH_3)_2$	4.27	3.98
15	2,5-$(CH_3)_2$	4.16	4.19
16	3,4-$(CH_3)_2$	3.88	4.04
17	3,5-$(CH_3)_2$	4.25	4.19
18	2,3,5-$(CH_3)_3$	4.22	4.44
19	2,4,5-Cl_3	4.71	4.39
20	Cl_5	4.64	5.01

[a] Calculated by Eq. [2].

comparison. For the *meta* substituent, a similar preference for molar refractivity is not observed. Taft's steric constant $E_s(m)$ and sterimol parameter for maximum width $B4(m)$ represented the steric effect of the *meta* substituent better than other parameters, showing that directional steric effect is involved. Equations [7] to [12] clarify this.

$$-\log EC_{50} = 3.636 + 0.992\,\Sigma\pi - 0.0079\,[MR(p)]^2 - 0.140\,(\Sigma\pi)^2 + 0.231\,\Sigma E_s(m) \quad [7]$$

$$n = 19,\ R = 0.922,\ s = 0.262,\ F = 19.83$$

$$-\log EC_{50} = 3.761 + 0.980\,\Sigma\pi - 0.0079\,[MR(p)]^2 - 0.123\,(\Sigma\pi)^2 - 0.063\,\Sigma MR(m) \quad [8]$$

$$n = 19,\ R = 0.920,\ s = 0.264,\ F = 19.35$$

$$-\log EC_{50} = 4.132 + 0.929\,\Sigma\pi - 0.0078\,[MR(p)]^2 - 0.083\,(\Sigma\pi)^2 - 0.030\,[\Sigma L(m)]^2 \quad [9]$$

$$n = 19,\ R = 0.916,\ s = 0.271,\ F = 18.17$$

$$-\log EC_{50} = 4.058 + 0.927\,\Sigma\pi - 0.0079\,[MR(p)]^2 - 0.080\,(\Sigma\pi)^2 - 0.108\,[\Sigma B1(m)]^2 \quad [10]$$

$$n = 19,\ R = 0.916,\ s = 0.271,\ F = 18.16$$

$$-\log EC_{50} = 4.275 + 0.988\,\Sigma\pi - 0.0079\,[MR(p)]^2 - 0.134\,(\Sigma\pi)^2 - 0.320\,\Sigma B2(m) \quad [11]$$

$$n = 19,\ R = 0.921,\ s = 0.262,\ F = 19.67$$

$$-\log EC_{50} = 4.188 + 0.992\,\Sigma\pi - 0.0079\,[MR(p)]^2 - 0.141\,(\Sigma\pi)^2 - 0.276\,\Sigma B4(m) \quad [12]$$

$$n = 19,\ R = 0.922,\ s = 0.262,\ F = 19.85.$$

It may also be noted that only equations with $[MR(p)]^2$ can explain the very low activity of *O,O*-bis(4-tertiary butyl phenyl) isopropyl phosphonate which was excluded from the regression analysis (Table 4), manifesting their predictive values. The development of Eq. [7] is presented elsewhere (8). The absence of the linear term of $MR(p)$ with a positive coefficient in the above equations shows that the optimum value of

TABLE 4
Calculated Values of -log EC_{50} (mol/liter) of 0,0-Bis(4-tertiary Butyl Phenyl) Isopropyl Phosphonate (Observed -log EC_{50} <2.59)

Equation	Calc value	Equation	Calc value	Equation	Calc value
[1]	3.66	[7]	2.01	[13]	1.47
[2]	1.92	[8]	2.05	[14]	1.57
[3]	3.91	[9]	2.13	[15]	4.84
[4]	3.06	[10]	2.11	[16]	4.11
[5]	3.78	[11]	2.02	[17]	4.29
[6]	5.53	[12]	2.01	[18]	1.10

MR(p) is about MR(p) = O. This means that hydrogen with the smallest MR value among common substituents (MR = 1.03) should be present at the *para* position. In other words, for the synthesis of more active compounds, the *para* position should preferably remain unsubstituted.

Pyricularia oryzae

Equation [13] gives the relationship between fungitoxicity of the series to *P. oryzae* and the chemical descriptors $\Sigma\pi$ and MR(p).

$$-\log EC_{50} = 3.282 + 0.405\,\Sigma\pi - 0.0068\,[MR(p)]^2 \quad [13]$$

$$n = 19,\ R = 0.788,\ s = 0.401,\ F = 13.10.$$

Substitution of MR(p) with Taft's parameter or sterimol parameters for the *para* substituent gave equations with poorer correlations as in *H. oryzae*. It was also found that while Eq. [13] could explain the very low activity of *O,O*-bis(4-tertiary butyl phenyl) isopropyl phosphonate which was excluded from the regression analysis (Table 4), other corresponding equations with Taft's parameter and sterimol parameters could not do so. The development of Eq. [13] is presented elsewhere (8). Equation [13] also shows that the optimum value of MR(p) is about MR(p) = O.

Alternaria alternata

Regression Eq. [14] gives the relationship between fungitoxicity to *A. alternata* and the chemical descriptors $\Sigma\pi$ and MR(p).

$$-\log EC_{50} = 3.066 + 0.356\,\Sigma\pi - 0.0057\,[MR(p)]^2 \quad [14]$$

$$n = 18,\ R = 0.784,\ s = 0.350,\ F = 11.97.$$

Substitution of MR(p) with sterimol parameters for the *para* substituent gave equations with poorer correlations. However, substitution of MR(p) with $E_s(p)$ gave an equation with slightly better correlation presumably because of the intercorrelation between $E_s(p)$ and other parameters. It may also be noted that while Eq. [14] could explain the very low activity of *O,O*-bis(4-tertiary butyl phenyl) isopropyl phosphonate which was excluded from the regression analysis (Table 4), corresponding equations with Taft's parameter and sterimol parameters could not explain it as in *H. oryzae*. Development of Eq. [14] is presented elsewhere (8). Equation [14] shows that the optimum value of MR(p) is about MR(p) = O.

Fusarium solani

Equation [15] gives the relationship between fungitoxicity to *F. solani* and the chemical descriptors $\Sigma\pi$ and MR(p).

$$-\log EC_{50} = 2.692 + 0.699\,\Sigma\pi - 0.140\,(\Sigma\pi)^2 + 0.067\,MR(p) \quad [15]$$

$$n = 9,\ R = 0.960,\ s = 0.176,\ F = 19.58.$$

Substitution of $MR(p)$ with $E_s(p)$, $B1(p)$, $B2(p)$, and $B4(p)$ gave equations with poorer correlations. However, substitution with $L(p)$ gave Eq. [16] with slightly better correlation.

$$-\log EC_{50} = 2.246 + 0.711\,\Sigma\pi - 0.147\,(\Sigma\pi)^2 + 0.251\,L(p) \quad [16]$$

$$n = 9,\ R = 0.961,\ s = 0.174,\ F = 20.06.$$

Although the statistical values of Eq. [15] and [16] are quite satisfactory, these equations are not quite reliable since they cannot explain the low activity ($EC_{50} > 1$ mg/ml) of any of the compounds excluded from the regression study. Since the number of compounds is only nine, all steric parameters are not well represented by these equations. Those compounds having bulky substituents (Cl, NO_2, SCH_3, C-$(CH_3)_3$) which showed very low activity ($EC_{50} > 1$ mg/ml) were excluded from the regression analysis. This is presumably the reason for the positive coefficient with $MR(p)$, unlike other fungi studied. Development of Eq. [15] is presented elsewhere (8).

Rhizoctonia bataticola

Unlike other test fungi studied, steric effect of a substituent at the *para* position of the phenyl ring was not found to play a dominant role here. Assuming the theory of receptor fit mechanism, this may be due to the fact that the cleft of the bioreceptor is broad enough to accommodate bulky substituents such as NO_2 and SCH_3. This belief is strengthened by the fact that *O,O*-bis(4-tertiary butyl phenyl) isopropyl phosphonate which has a bulkier substituent at the *para* position did not give measurable EC_{50} < 1 mg/ml and was thus eliminated from the regression analysis.

Equation [17] gives the relationship between fungitoxicity to *R. bataticola* and chemical descriptors, namely $\Sigma\pi$, $\Sigma B4(m)$, and $\Sigma F'$.

$$-\log EC_{50} = 3.590 + 0.667\,\Sigma\pi - 0.171\,(\Sigma\pi)^2 + 0.461\,\Sigma F' + 0.013\,[(\Sigma B4(m)]^2 \quad [17]$$

$$n = 19,\ R = 0.977,\ s = 0.074,\ F = 72.68.$$

Substitution of $\Sigma B4(m)$ with $\Sigma MR(m)$ and other sterimol parameters for *meta* substituents gave equations with poorer correlations. Use of Taft's parameter $\Sigma E_s(m)$ in lieu of $\Sigma B4(m)$ also gave an equation with poorer correlation, but it gave an equation with better correlation than that with $\Sigma MR(m)$. This shows that the steric effect of the *meta* substituent is directional in nature. Development of the corresponding equation with $\Sigma E_s(m)$ in place of $\Sigma B4(m)$ is presented elsewhere (7).

Sclerotium rolfsii

Equation [18] gives the relationship between fungitoxicity to *S. rolfsii* and the chemical descriptors $\Sigma\sigma$ and $MR(p)$.

$$-\log EC_{50} = 3.166 + 0.304\,\Sigma\sigma - 0.012\,[MR(p)]^2 + 0.133\,MR(p) \quad [18]$$

$$n = 19,\ R = 0.654,\ s = 0.354,\ F = 3.74.$$

Substitution of $MR(p)$ with Taft's parameter and sterimol parameters for *para* substituents gave equations with poorer correlations.

Although the statistical values of Eq. [18] are not satisfactory, it explains the very low activity of *O,O*-bis(4-tertiary butyl phenyl) isopropyl phosphonate, which was excluded from the regression analysis (Table 4). Equation [18] allows us to calculate the

optimum value of MR(p) as 5.5. Development of Eq. [18] is presented elsewhere (9).

DISCUSSION

Molar refractivity represents the steric effect of the substituent at the *para* position of the phenyl ring better than Taft's steric constant and sterimol parameters showing that bulk tolerance factor rather than the directional steric effect is involved. The parabolic relationship between molar refractivity of the *para* substituent and fungitoxicity has been established in the cases of four fungi studied. Presuming the theory of receptor fit mechanism (3), this shows that there is an optimum bulk of the *para* substituent by which it will fit into the narrow cleft of the bioreceptor. In the case of some fungi (*A. alternata, H. oryzae,* and *P. oryzae*), the cleft of the bioreceptor may be so small that even hydrogen has a slightly higher bulk than the optimum bulk. This may be the reason for the absence of a linear term of MR(p) with the positive coefficient in the corresponding regression equations. In the case of *R. bataticola,* the cleft may be broad enough to fit bulky substituents such as NO_2 and SCH_3. The cases of *F. solani* and *S. rolfsii* may lie between these extremes. Steric effects of substituents at the *para* position of the phenyl ring can be visualized as intermolecular only. Hence, the results indicate that MR represents intermolecular steric effects better than other parameters.

Steric effects of substituents at the *meta* position of the phenyl ring played comparatively minor but statistically significant roles in determining the fungitoxicity of the series to two fungi. Either Taft's steric constant or the sterimol parameter for maximum width gives a better correlation, showing that they are directional in nature.

The biological or biochemical function inhibited by *O,O*-bisaryl isopropyl phosphonates is not known. However, permeation and transport to the receptor site must be largely governed by lipophilicity of the compounds. Once the compounds reach the site of action, interaction with the receptor must be governed by steric considerations, especially that of the *para* substituent. It is also noteworthy that there is no significant intercorrelation between $\Sigma\pi$ and MR(p) ($r = -0.02$) and the effects of the two are distinct. The compounds must fit into the cleft of the bioreceptor at the *para* position of the phenyl ring (Fig. 1). Once they fit into the receptor, what happens to the compounds is not known. Their probable degradation products, isopropyl phosphonic acid and phenols, may themselves act as fungitoxicants. It may be noted that phosphonic acid possesses fungitoxicity (11) and is supposed to be the toxophore of the well-known fungicide Aliette (12). Phenols, especially chlorophenols, are fungitoxic. It is also known that fungitoxicity of chlorophenols is largely governed by lipophilicity (13). The reason for the poorer fungitoxicity of phosphonic acid and phenols compared to *O,O*-bisaryl isopropyl phosphonates should be their poorer lipophilicity due to the presence of polar OH groups.

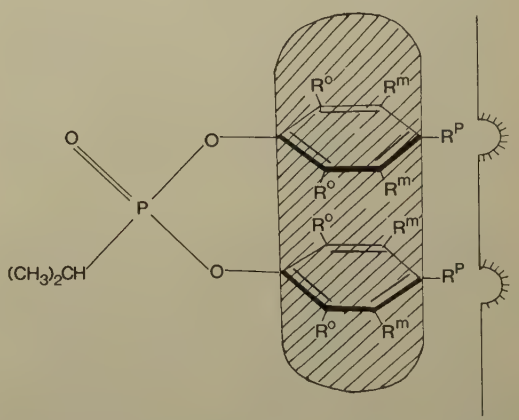


FIG. 1. *Receptor diagram of O,O-bisaryl isopropyl phosphonates: Substituents at* ortho, meta, *and* para *positions are designated as* R^o, R^m, *and* R^p, *respectively. The shaded area shows lipophilic sites which facilitate the permeation and transport of the compounds to the bioreceptor. Lines with fringes represent three-dimensional steric interaction sites of the receptor to which molecules must fit.*

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Malathion Antagonizes Metabolism-Based Chlorsulfuron Resistance in *Lolium rigidum*

JOHN T. CHRISTOPHER, CHRISTOPHER PRESTON, AND STEPHEN B. POWLES[1]

Department of Crop Protection, Waite Agricultural Research Institute, University of Adelaide, P.B.M.1, Glen Osmond, South Australia, 5064

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A biotype of *Lolium rigidum* Gaud. (SLR31) is resistant to the sulfonylurea herbicide chlorsulfuron, despite having a herbicide-sensitive target site, acetolactate synthase. This biotype is able to metabolize the herbicide at a faster rate than a susceptible biotype. Seedlings of this biotype treated with chlorsulfuron in combination with the organophosphate insecticide malathion exhibited greatly increased mortality and reduced dry weight compared to seedlings treated with chlorsulfuron alone. The chlorsulfuron LD_{50} for resistant biotype SLR31 decreased from 293.5 g ai ha^{-1} in the absence of malathion to 84.6 g ai ha^{-1} in the presence of 1000 g ai ha^{-1} malathion. The LD_{50} for a susceptible biotype was also reduced from 7.6 g ai ha^{-1} in the absence of malathion to 0.9 g ai ha^{-1}. Excised seedlings of the resistant biotype metabolized [phenyl-U-^{14}C]chlorsulfuron in the culm tissue nearest the meristem faster than the susceptible biotype. However, when the herbicide was given in combination with malathion, metabolism was dramatically reduced in both biotypes. In seedlings of the resistant biotype given [phenyl-U-^{14}C]chlorsulfuron alone 83.5 ± 2.3% of the herbicide taken into the culms tissue was metabolized after 9 hr. However, when the herbicide was given in combination with 70 μM malathion, only 13.0 ± 2.2% [phenyl-U-^{14}C]chlorsulfuron was metabolized after 9 hr. Thus, malathion increases chlorsulfuron toxicity for *L. rigidum* by inhibiting herbicide metabolism. As malathion has previously been shown to inhibit cytochrome P450-dependant monooxygenase-catalyzed primisulfuron metabolism by *Zea mays* microsomes, this result supports the hypothesis that chlorsulfuron metabolism in *L. rigidum* may be mediated by a cytochrome P450 isozyme. Other cytochrome P450 inhibitors, piperonylbutoxide and tetcyclasis, did not increase chlorsulfuron toxicity for either resistant or susceptible *L. rigidum* biotypes, while 1-aminobenzotriazole caused only a small increase in mortality and a small reduction in [^{14}C]chlorsulfuron metabolism in the resistant biotype. © 1994 Academic Press, Inc.

INTRODUCTION

Pest organisms from many orders have developed resistance to chemical control, with the widespread development of insecticide resistance of particular concern (1). The development of herbicide resistance in weed species has become a major problem more recently [see (2)]. Organisms may use a variety of behavioral and/or physiological mechanisms to avoid the toxic effects of xenobiotic control agents. Increased ability to detoxify xenobiotics has been widely reported as a mechanism of resistance, especially in cases of insecticide resistance (1). Often insecticide detoxification is mediated by cytochrome P450-dependant monooxygenases (cyt P450s),[2] particularly in cases of organophosphate, pyrethroid, and DDT resistance (1). In some instances, cyt P450-mediated detoxification causes cross-resistance to chemical classes of insecticides to which the insect population has not been exposed (3). Resistance of several classes of insecticides in a single species may result from increased detoxification by multiple cyt P450 enzyme systems (4). Inhibitors of insect cyt P450s have been widely used to increase the efficacy of certain insecticides (5).

[1] To whom correspondence should be addressed.

[2] Abbreviations used: ABT, 1-aminobenzotriazole; ALS, acetolactate synthase (EC 4.1.3.18); ai, active ingredient; LD_{50}, herbicide dose required to kill 50% of a population; cyt, cytochrome; PBO, piperonylbutoxide; PS II, photosystem II.

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Cyt P450s are membrane-bound enzyme systems containing heme-coordinated divalent cations which facilitate the oxidation of a wide range of substrates using reducing power from NADH or NADPH (6). They are required for many metabolic processes and are involved in the detoxification of xenobiotics in the mammalian liver, insects, fungi, and plants [reviewed in (7)]. Many different cyt P450 isozymes occur in the various tissues and organs of animals and plants (6), as well as being differentiated between the organelles of individual cells (8). There is increasing evidence for the occurrence of many different isozymes of cyt P450s in plant tissues which differ in level and range of substrate specificity and level of inducibility or inhibition by exogenous chemicals (8).

Microsomal cyt P450s capable of metabolizing herbicides have been isolated from several crop species and are often associated with herbicide insensitivity in these species [reviewed in (9)]. Wheat microsomal cyt P450s with the capacity to metabolize diclofop-methyl, chlorotoluron, 2,4-dichlorophenoxyacetic acid, chlorsulfuron, and triasulfuron have been reported (10–14). The cyt P450 inhibitors, piperonylbutoxide (PBO), 1-aminobenzotriazole (ABT), and tetcyclasis, can enhance the phytotoxicity of chlorotoluron toward wheat (15). PBO, tetcyclasis, paclobutrazol, and tridiphane have also been shown to inhibit chlorotoluron detoxification by wheat microsomal cyt P450s (10). Maize is another crop in which cyt P450-dependant metabolism is important for herbicide insensitivity. Maize microsomal cyt P450s are able to metabolize primisulfuron. Soil-applied insecticides inhibit primisulfuron metabolism in maize causing crop damage (16, 17).

Although cyt P450-mediated metabolism is a common mechanism of insecticide resistance and of herbicide selectivity in crop species, the evolution of herbicide resistance due to increased herbicide metabolism has been rarely reported. A biotype of *Abutilon theophrasti* has evolved the ability to rapidly metabolize the herbicide atrazine but in this case glutathione transferase catalyzes the initial step in detoxification (18). Rapid metabolism has also been suggested as the mechanism of resistance for chlorotoluron-resistant biotypes of *Alopecurus myosuroides* (19). The cyt P450 inhibitor ABT can increase chlorotoluron toxicity for these *A. myosuroides* biotypes, suggesting that cyt P450s are involved in detoxification (19).

Increased herbicide metabolism has been reported as a resistance mechanism for some herbicide-resistant biotypes of *Lolium rigidum* in Australia. Multiple-resistant *L. rigidum* biotype SLR31 is cross-resistant to several sulfonylurea and imidazolinone herbicides despite having a herbicide-sensitive target enzyme, acetolactate synthase (ALS) (EC 4.1.3.18) (20). Resistance to the wheat-selective ALS inhibitor chlorsulfuron in biotype SLR31 involves increased herbicide metabolism by a mechanism similar to that operating in wheat (21, 22). The major metabolite of chlorsulfuron metabolism in *L. rigidum* coeluted during high-pressure liquid chromatography (HPLC) analysis with the major metabolite of chlorsulfuron produced by wheat (21). The major metabolite in wheat has been identified as a sugar conjugate of hydroxychlorsulfuron, formed following hydroxylation of chlorsulfuron in the phenyl ring (23). Cotterman and Saari (22) confirmed that the major product of chlorsulfuron metabolism in *L. rigidum* could be hydrolyzed using the enzyme β-glucosidase to give a product which coeluted with an hydroxychlorsulfuron standard. The production of such an aryl-hydroxylated intermediate is a reaction typical of xenobiotic metabolism mediated by cyt P450s (7, 9) and formation of this product by microsomal cyt P450s from wheat has been demonstrated (12).

Other *L. rigidum* biotypes are resistant to certain photosystem II (PS II)-inhibiting herbicides, including chlorotoluron and

simazine, despite having a herbicide-sensitive PS II. Resistance in these biotypes is most likely to be due to increased metabolism mediated by cyt P450 (24, 25). The cyt P450 inhibitors ABT and PBO increased chlorotoluron toxicity and ABT dramatically reduced metabolism of [^{14}C]chlorotoluron in these *L. rigidum* biotypes (24).

Current understanding of the role of cyt P450-catalyzed metabolism in herbicide resistance in *L. rigidum* is limited by a lack of detectable levels of herbicide metabolism in microsomes isolated from this species (Preston and Powles, unpublished data, 1993). Here, however, we report evidence from *in vivo* studies, using known cyt P450 inhibitors, which strongly suggests that cyt P450-catalyzed metabolism is involved in resistance to some herbicides for certain *L. rigidum* biotypes.

MATERIALS AND METHODS

Chemicals

The organophosphate insecticide malathion is known to inhibit cyt P450-mediated metabolism of a sulfonylurea herbicide in *Zea mays* (16). Emulsification of malathion was achieved using hydrocarbon solvent and nonionic surfactant emulsifier as used for commercial formations of malathion (Incitec Pty Ltd, Cavan, South Australia). The same combination of solvent and emulsifier was used with PBO. Commercial formulations of herbicides used for whole plant studies were supplied by DuPont (Australia) Ltd and Ciba-Geigy Australia Ltd. [phenyl-U-^{14}C]chlorsulfuron (11.5 μCi/mg) was supplied by DuPont Agricultural Products (Newark, DE).

Plant Material

One susceptible and three herbicide-resistant biotypes of *L. rigidum* were used (Table 1). The cyt P450 inhibitor ABT has been previously shown to synergize the toxicity of the PS II inhibitors chlorotoluron and simazine for the metabolism-based resistant biotype WLR2 (24, 25). This biotype was used as a positive control for the effect of P450 inhibitors in whole plant studies. The chlorsulfuron-insensitive crop species wheat (*Triticum aestivum* cv Machete) was also tested.

L. rigidum seedlings were germinated on 0.6% (w/v) agar-solidified medium. Approximately 7 days later, seedlings of equal size and vigor were transplanted to either potting medium or a hydroponic system. Wheat seed were sown directly into potting soil at the time of transplanting ryegrass seedlings to produce seedlings of wheat and *L. rigidum* of equal two-leaf growth stage at the time of treatment.

Response of Pot-Cultured Plants to Herbicides in Combination with Malathion and PBO

L. rigidum seedlings germinated and transplanted, as described above, were placed into 2-liter pots containing potting soil (21 plants per pot) kept outdoors during the normal autumn-winter growing season. Herbicides were applied in a laboratory

TABLE 1

Herbicide Resistance Status and Known Resistance Mechanisms of Lolium rigidum Biotypes Used in This Study

Biotype	Resistance status	Resistance mechanisms	References
VLR1	Susceptible	—	—
SLR31	Chlorsulfuron	Enhanced metabolism	21, 22
	Also to many other herbicide groups	Varied	30
WLR1	ALS-inhibiting herbicides	Insensitive ALS	28
WLR2	PS II-inhibiting herbicides	Enhanced metabolism	24, 25

spraying cabinet, 7 to 14 days after transplanting, when plants were at the two-leaf stage as previously described (21). Plants were placed in the field in a randomized complete block design using 21 plants per treatment with four replicates of each treatment. Plants were harvested and scored for mortality 4 or 5 weeks after treatment. Plants with live meristems which had clearly increased in size since the time of spraying were scored as alive and harvested at soil level, dried at 80°C for 48 hr, then weighed. In all cases the trends apparent in dry weight data reflected those in data for mortality, unless otherwise stated. Therefore, only mortality data are presented.

For malathion experiments, malathion was applied at the rate of 0, 63, 250, or 1000 g active ingredient (ai) ha^{-1} immediately prior to herbicide application. Biotypes VLR1, SLR31, and WLR1 were treated with chlorsulfuron at rates of 1, 2, 4, 8, 16, 64, 256, 512, and 1024 g ai ha^{-1}. Biotypes WLR2 and VLR1 were treated with rates of 750, 1500, 3000, and 6000 g ai ha^{-1} chlorotoluron. Probit analysis was used to calculate LD_{50} values in the presence and absence of malathion using the computer program Polo PC.

PBO experiments were conducted in a similar manner to the malathion experiments described above except that PBO was applied in two applications of 2100 g ai ha^{-1} in 97 liter ha^{-1} water to give a total application of 4200 g ai ha^{-1} in a total volume of 194 liter ha^{-1} immediately prior to herbicide application.

Control plants treated with herbicide plus the commercial solvent and emulsifier used to formulate malathion and PBO for spray application were not significantly different from plants treated with the herbicide alone (data not shown). Thus, any interactive phytotoxic effects between spray applications of herbicide in combination with malathion or PBO are not due to effects of the emulsifier and/or surfactant on the cuticle and/or phospholipid membranes (26).

Response of Hydroponics-Cultured Plants to Herbicides in Combination with ABT and Tetcyclasis

As the water-soluble cyt P450 inhibitors ABT and tetcyclasis are not readily absorbed via the leaf surface, they were added to the nutrient solution bathing the roots of plants grown in hydroponics. Seedlings, germinated as above, were transferred to 250 ml polycarbonate vials containing nutrient medium based on that of Hoagland (27). Twenty-one plants per vial were supported by fiberglass mesh with the roots immersed in the nutrient medium and shoots extending through 0.5-cm holes in a blackened plastic vial lid. Vials were wrapped with aluminum foil to prevent light reaching the roots. Nutrient solution was changed at the time of treatment (when plant were at the two-leaf stage) after which the solution was regularly replenished with nutrient solution to replace transpiration losses. Control vials received fresh nutrient solution only. Mortality, dry weight of roots, and dry weight of shoots were determined 21 days after treatment. In all cases the trends apparent in dry weight data reflected those in mortality data unless otherwise stated. Pilot experiments were used to determine the maximum levels of each inhibitor which could be applied without causing phytotoxic effects in the absence of herbicide. For ABT experiments using *L. rigidum* biotypes VLR1, WLR1, and SLR31, nutrient solution was replaced at the two-leaf stage with fresh nutrient solution only or solution containing 70 μM ABT, 100 nM chlorsulfuron, or 70 μM ABT plus 100 nM chlorsulfuron. Four replicate vials for each treatment were used for the ABT experiment. Data were analyzed by two-way analysis of variance. Tetcyclasis experiments were similar to those described for ABT except that seedlings of *L. rigidum* biotype WLR2 were also treated using 3.7 μM tetcyclasis, herbicide alone, or 3.7 μM tetcyclasis plus herbicide. For biotypes VLR1, SLR31, and WLR1, 100

n*M* chlorsulfuron herbicide was used. For biotype WLR2, 2 μ*M* chlorotoluron was used. The tetcyclasis experiment was replicated three times.

[^{14}C]*Chlorsulfuron Metabolism in Combination with cyt P450 Inhibitors*

L. rigidum seedlings germinated and transplanted into potting soil, as described above, were grown in a growth cabinet (22°C, 14 hr, 280–320 μmol photons m^{-2} sec^{-1} light period, 15°C, 10 hr dark period). Seedlings were excised at soil level 14 days after transplanting when at the two-leaf stage and the bases of the seedlings placed in vials containing 6 μg/ml [phenyl-U-^{14}C]-chlorsulfuron (11.5 μCi/mg, supplied by DuPont) plus or minus cyt P450 inhibitors. PBO, ABT, malathion, and tetcyclasis were all used at 70 μ*M*. After 3 hr exposure to herbicide solution, plants were either harvested or placed into vials of distilled water for various periods before harvest. Treatment conditions and extraction were as previously described (28), leaf lamina were separated from the lower portion of the shoots (culms) for separate extraction and HPLC analysis. The culms consisted of the sheath of the first leaf and the section of the second leaf enclosed therein. Reverse-phase HPLC analysis of [^{14}C]chlorsulfuron and metabolites was conducted as previously described (28).

RESULTS

Whole Plant Response to Spray Applications of Herbicides in Combination with cyt P450 Inhibitors

Response to herbicides in combination with malathion. The response of pot-cultured plants treated by spray applications of chlorsulfuron in the presence and absence of malathion is shown in Fig. 1. Treatment with malathion alone at up to 1000 g ai ha^{-1} did not cause mortality of either the resistant biotype SLR31 or the susceptible biotype (Fig. 1). However, malathion in the presence of chlorsulfuron

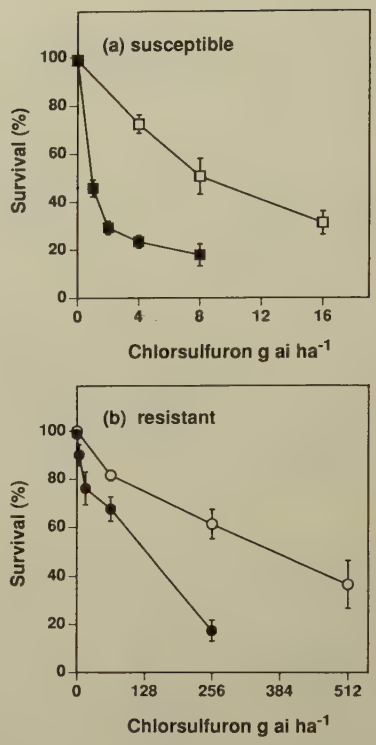

FIG. 1. *Survival, 35 days after treatment, of pot grown L. rigidum plants of susceptible biotype VLR1 (a) treated with chlorsulfuron in the presence (■) or absence (□) of 1000 g ai ha^{-1} malathion and of metabolism-based resistant biotype SLR31 (b) in the presence (●) or absence of malathion (○). Vertical bars represent the standard error of the mean of four replicates.*

greatly increased mortality for both biotypes (Fig. 1). Probit analysis reveals the LD_{50} for chlorsulfuron was reduced for both biotypes when herbicide was given in the presence of 250 or 1000 g ai ha^{-1} malathion (Table 2). Although the LD_{50} for resistant biotype SLR31 was substantially reduced by the addition of 1000 g ai ha^{-1} malathion (Table 2) it did not approach the LD_{50} of the susceptible biotype VLR1.

Results for biotype WLR1, which is resistant to ALS inhibitors due to herbicide-insensitive ALS (28), contrast with those for the metabolism-based resistant biotype SLR31. Malathion did not counter chlorsulfuron resistance in the target site mutant WLR1. Even when treated with the high

TABLE 2

Chlorsulfuron LD_{50}'s (g ai ha ^{1}) with 95% Confidence Intervals (CI) for Resistant and Susceptible L. rigidum Biotypes Treated with Chlorsulfuron in the Presence or Absence of Malathion

Malathion (g ai ha^{-1})	Susceptible (VLR1)		Chlorsulfuron-resistant (SLR31)	
	LD_{50} (g ai ha^{-1})	CI (0.95) (lower to upper limit)	LD_{50} (g ai ha^{-1})	CI (0.95) (lower to upper limit)
0	7.6	5.7 to 10.0	293.5	212.0 to 404.6
63	7.6	5.7 to 10.2	314.6	227.5 to 434.7
250	4.6	3.4 to 6.2	167.3	119.5 to 236.3
1000	0.9	0.7 to 1.3	84.6	57.3 to 126.9

Note. Values calculated by probit analysis using data from experiments partially represented in Fig. 1.

dose of 512 g ai ha^{-1} chlorsulfuron, biotype WLR1 exhibited 1.3 ± 1.3% mortality in the absence of malathion and this only increased to 3.8 ± 1.2% in the presence of 250 g ai ha^{-1} malathion. This small difference was not statistically significant ($P > 0.05$). Thus, the malathion-induced increase in chlorsulfuron toxicity was observed only for the biotypes with herbicide-sensitive ALS which are largely dependant upon herbicide metabolism to avoid phytotoxic effects.

HPLC analysis of the products of chlorsulfuron metabolism in *L. rigidum* suggests that the metabolic pathway in *L. rigidum* is similar to that in wheat (21, 22). However, the rate of chlorsulfuron metabolism in wheat shoots is more rapid than that in the chlorsulfuron-resistant biotype SLR31 (21). Application of chlorsulfuron at rates up to 512 g ai ha^{-1} caused little mortality of wheat in either the presence or absence of 250 g ai ha^{-1} malathion (data not shown). Thus, any interactive effects between malathion and chlorsulfuron are not sufficient to cause significant mortality in wheat at these rates. As the inherent rate of chlorsulfuron metabolism in wheat is faster than in *L. rigidum* (21), higher levels of malathion may be required to cause increased mortality. This result suggests that malathion or less toxic analogues might be used to increase the efficacy of chlorsulfuron for *L. rigidum* control while retaining selectivity in wheat.

Response to herbicides in the presence of PBO. In contrast to malathion (Fig. 1), spray application of the commercial insecticide synergist PBO in combination with chlorsulfuron did not increase mortality of chlorsulfuron-resistant biotype SLR31 (Fig. 2). For the metabolism-based chlorotoluron-resistant biotype WLR2, however, 4200 g ai ha^{-1} PBO in combination with chlorotoluron did increase mortality to a level similar to that of a susceptible biotype (not shown). The interactive effect of PBO and chlorotoluron for biotype WLR2 indicates that PBO can interact with herbicides when applied to *L. rigidum* in this system.

Whole Plant Response to Applications of Herbicides in Combination with cyt P450 Inhibitors in Hydroponic Nutrient Solution

Response to ABT. There was some inter-

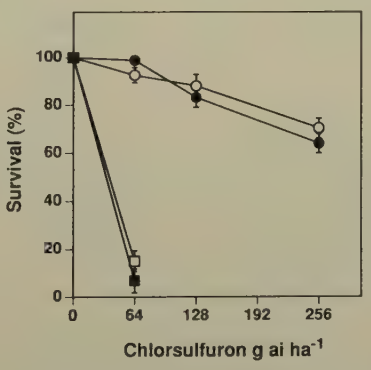

FIG. 2. *Survival, 35 days after treatment, of pot grown L. rigidum biotype SLR31 treated with chlorsulfuron in the presence (●) or absence (○) of 4200 g ai ha^{-1} PBO and susceptible biotype VLR1 in the presence (■) or absence (□) of PBO. Vertical bars represent the standard error of the mean of four replicates.*

action between the effects of hydroponically-applied chlorsulfuron and ABT upon chlorsulfuron-resistant biotype SLR31. ABT applied alone had no detectable phytotoxic effect for any *L. rigidum* biotype tested (Table 3). Although shoot dry weight of surviving plants was not significantly affected (data not shown) there was an interactive effect on mortality caused by ABT plus chlorsulfuron (Table 3). However, the increased mortality caused by ABT plus chlorsulfuron applied to SLR31 (27 ± 6.6%) was considerably less than the mortality caused by chlorsulfuron alone applied to the susceptible VLR1 (82 ± 5.7%) (Table 3).

The interaction between the effects of chlorsulfuron and ABT on mortality was not significant for the susceptible biotype or the ALS mutant WLR1 (Table 3). Thus, the interactive effect between ABT and chlorsulfuron is only observed for biotype SLR31 which rapidly metabolizes chlorsulfuron but has a herbicide-sensitive ALS.

The results of Burnet *et al.* (24), using a similar hydroponic system, demonstrated that 70 μ*M* ABT strongly synergized chlorotoluron phytotoxicity for chlorotoluron-resistant biotype WLR2 (24), indicating that ABT can interact with herbicides when applied via the hydroponic nutrient solution.

Response to tetcyclasis. Addition of tetcyclasis did not increase chlorsulfuron toxicity for any *L. rigidum* biotypes (Table 4). Tetcyclasis did increase chlorotoluron toxicity for biotype WLR2 when applied via the hydroponic nutrient medium (Table 4). The synergistic effect of tetcyclasis and chlorotoluron for biotype WLR2 indicates that tetcyclasis can interact with herbicides when applied in this manner.

[^{14}C]*Chlorsulfuron Metabolism*

Excised seedlings of chlorsulfuron-resistant biotype SLR31 rapidly metabolized [^{14}C]chlorsulfuron in the culm tissue in the absence of malathion, metabolizing 50% of the herbicide in approximately 4.5 hr (Fig. 3). This rate of metabolism is in good agreement with previous results (28). However, when [^{14}C]chlorsulfuron was given in combination with 70 μ*M* malathion, metabolism was dramatically reduced. [^{14}C]chlorsulfuron metabolism was reduced from 83.5 ± 2.3% after 9 hr in the absence of malathion to 13.3 ± 2.2% in the presence of malathion (Fig. 3). Metabolism of chlorsulfuron in the absence of malathion in the leaf lamina was much slower than that in culms, as has been previously reported (28) (data not shown). Regression analysis reveals that [^{14}C]chlorsulfuron metabolism in the presence of malathion is significantly inhibited for both the leaf lamina and the culm tissue ($P < 0.05$). Metabolism of [^{14}C]chlorsulfuron in tissues of the susceptible biotype was also inhibited when

TABLE 3

Mortality (%) of Susceptible (VLR1), Metabolism-Based Chlorsulfuron-Resistant (SLR31), and ALS-Based Chlorsulfuron-Resistant (WLR1) Biotypes of L. rigidum Grown in Hydroponics 30 Days after Commencement of Treatment with 100 nM Chlorsulfuron in the Presence or Absence of the cyt P450 Inhibitor ABT at a Final Concentration of 70 μM in the Nutrient Medium

	Mortality (%)			
Biotype	Untreated	70 μ*M* ABT	100 n*M* Chlorsulfuron	100 n*M* Chlorsulfuron + 70 μ*M* ABT
VLR1	2.6 ± 2.6	0 ± 0	81.6 ± 5.7	95.2 ± 4.8*
SLR31	0 ± 0	1.8 ± 1.8	6.3 ± 3.9	26.5 ± 3.8**
WLR1	6.5 ± 0.6	7.5 ± 3.8	3.9 ± 2.0	7.9 ± 0.9*

Note. Data represent the mean and standard error of four replicate experiments. Two-way analysis of variance indicates that the interaction between inhibitor and herbicide effects is not significant (*$P > 0.05$) or is significant (**$P < 0.05$).

TABLE 4

Mortality (%) of Susceptible (VLR1), Metabolism-Based Chlorsulfuron-Resistant (SLR31), ALS-Based Chlorsulfuron-Resistant (WLR1), and Metabolism-Based Chlorotoluron-Resistant (WLR2) Biotypes of L. rigidum Grown in Hydroponics 30 Days after Commencement of Treatment with Herbicide in the Presence or Absence of the cyt P450 Inhibitor Tetcyclasis at a Final Concentration of 3.7 μM in the Nutrient Medium

Biotype	Herbicide concentration	Mortality (%)			
		Untreated	Tetcyclasis alone	Herbicide alone	Herbicide + tetcyclasis
VLR1	Chlorsulfuron 100 n*M*	2.0 ± 2.0	21.0 ± 9.5	89.8 ± 5.8	89.2 ± 3.2*
SLR31	Chlorsulfuron 100 n*M*	0 ± 0	9.1 ± 1.8	18.5 ± 3.7	11.8 ± 7.2*
WLR1	Chlorsulfuron 100 n*M*	3.3 ± 1.6	3.5 ± 1.8	10.4 ± 2.5	13.7 ± 4.4*
WLR2	Chlorotoluron 2 m*M*	10.3 ± 5.1	7.2 ± 4.9	37.1 ± 10.3	86.4 ± 4.0**

Note. Data represent the mean and standard error of three replicate experiments. Two-way analysis of variance indicates that the interaction between the effects of inhibitor and herbicide is not significant (*$P > 0.05$) or is significant (**$P < 0.05$).

[^{14}C]chlorsulfuron was given in combination with 70 μ*M* malathion. After 6 hr, 38.5 ± 4.1% of [^{14}C]chlorsulfuron in the culms was metabolized in the absence of malathion, while metabolism in the presence of malathion was not detectable.

Data for [^{14}C]chlorsulfuron metabolism after 3 hr in the culms of metabolism-based chlorsulfuron-resistant SLR31 seedlings in the presence of the cyt P450 inhibitors PBO, tetcyclasis, and ABT are given in Fig. 4. None of these three cyt P450 inhibitors were able to prevent substantial metabolism of [^{14}C]chlorsulfuron after 3 hr, although malathion completely inhibited metabolism within this period (Fig. 3). Tetcyclasis had no detectable effect, while metabolism of [^{14}C]chlorsulfuron in the presence of ABT was slightly lower than that of [^{14}C]chlorsulfuron alone (Fig. 4). Uptake of [^{14}C]chlorsulfuron did not differ significantly between treatments (data not shown).

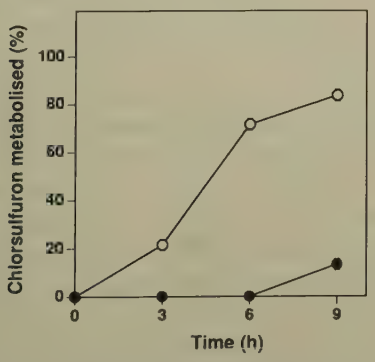

FIG. 3. [^{14}C]*Chlorsulfuron metabolized in culms of metabolism-based chlorsulfuron-resistant L. rigidum biotype (SLR31) in the presence (●) and absence (○) of 70 μM malathion, expressed as percentage of total [^{14}C] extracted. Vertical bars represent the standard error of the mean of four replicates.*

DISCUSSION

The organophosphate insecticide malathion increased chlorsulfuron toxicity for

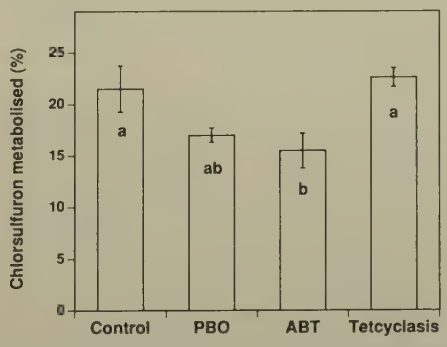

FIG. 4. [^{14}C]*Chlorsulfuron metabolized after 3 hr in culms of a metabolism-based chlorsulfuron-resistant biotype SLR31 in the absence of inhibitors (control) or in the presence of 70 μM PBO, ABT, or tetcyclasis, expressed as percentage of total* [^{14}C] *extracted from each tissue. Metabolism in the presence of 70 μM malathion over this period was undetectable (Fig. 3). Vertical bars represent the standard error of the mean of four replicates. Bars containing the same letter are not significantly different ($P > 0.05$; LSD = 4.9).*

both the metabolism-based chlorsulfuron-resistant *L. rigidum* biotype SLR31 and a susceptible biotype (Fig. 1, Table 2) and almost completely inhibited metabolism of chlorsulfuron in excised *L. rigidum* seedlings (Fig. 3). These results provide further evidence that increased metabolism of chlorsulfuron is an important factor for chlorsulfuron resistance in biotype SLR31. Malathion has been previously shown to inhibit the metabolism of primisulfuron by microsomal cyt P450-dependant monooxygenases from *Z. mays* (16). Primisulfuron is a sulfonylurea herbicide with structural similarity to chlorsulfuron. Thus, these results suggest that chlorsulfuron metabolism in *L. rigidum* may be catalyzed by a cyt P450. HPLC data on the products of *L. rigidum* chlorsulfuron metabolism are also in agreement with this hypothesis (21, 22).

As malathion is oxidized by cyt P450s to the biologically active malaoxon in animals and plants (29) it has been suggested that the cyt P450 which oxidizes malathion may be the same one catalyzing primisulfuron hydroxylation in *Z. mays* (16). Similarly, it could be argued that chlorsulfuron metabolism in *L. rigidum* may be catalyzed by a cyt P450 which also catalyzes oxidation of malathion. This would lead to competition between malathion and chlorsulfuron for the binding site of the detoxifying enzyme. Enzyme kinetic studies are needed to test this hypothesis. The constitutive function, if any, of plant cyt P450s able to catalyze the sulfoxidation of malathion is not known. However, a cyt P450 with a separate, constitutive function which is also involved in herbicide metabolism in plants has been previously reported (14). Evidence suggests that the cyt P450 catalyzing diclofop-methyl hydroxylation in wheat may be the same as that which catalyzes lauric acid hydroxylation (14).

The effects of cyt P450 inhibitors ABT, PBO, and tetcyclasis on [^{14}C]chlorsulfuron metabolism for chlorsulfuron-resistant biotype SLR31 correlate with the effects on whole plants. The compound which caused the greatest increase in chlorsulfuron toxicity for whole plants is malathion (Fig. 1, Table 2) and malathion caused the highest level of inhibition of [^{14}C]chlorsulfuron metabolism (Figs. 3 and 4). PBO and tetcyclasis had little effect on either chlorsulfuron toxicity at the whole plant level (Fig. 2, Table 4) or on [^{14}C]chlorsulfuron metabolism (Fig. 4). ABT caused a small increase in mortality (Table 3) and a small decrease in [^{14}C]chlorsulfuron metabolism (Fig. 4).

The possibility that factors which affect uptake, translocation, and/or metabolism of the cyt P450 inhibitors may also affect whole plant response cannot be ruled out. However, the close correlation between the effects of the inhibitors ABT, PBO, and tetcyclasis upon [^{14}C]chlorsulfuron metabolism in excised stems and the response observed for whole plants suggests that such differences are not important in these cases. In contrast, the malathion data for whole plant response do not exactly correlate with the [^{14}C]chlorsulfuron metabolism as malathion is able to almost totally inhibit [^{14}C]chlorsulfuron metabolism in excised stems (Fig. 3), but application of malathion at up to 1000 g ai ha^{-1} cannot reduce the LD_{50} of chlorsulfuron-resistant biotype SLR31 to that of a susceptible biotype (Table 2). The rates of uptake and metabolism of malathion by resistant and susceptible *L. rigidum* are not known. This leaves open two distinct possible explanations for the lack of full synergism of chlorsulfuron by malathion in SLR31. First, the resistant biotype may be able to metabolize malathion at a rate which prevents total inhibition of chlorsulfuron degradation. This would be expected if malathion degradation is catalyzed by the same enzyme system which degrades chlorsulfuron and is, therefore, increased in the resistant biotype. Alternatively, metabolism which can be inhibited by malathion may not be the only mechanism of chlorsulfuron resistance in biotype SLR31. This biotype has been shown to have multiple resistance mechanisms for several groups of chemically dissimilar her-

bicides [see (30)] and may also do so for chlorsulfuron. Further studies are required to clarify the apparent discrepancy between the whole plant response of biotype SLR31 to chlorsulfuron in combination with malathion and the [^{14}C]chlorsulfuron metabolism in excised stems.

These results also raise questions about differences in herbicide metabolism between *L. rigidum* biotypes with different resistance patterns. The results of whole plant studies indicate a clear difference in response to cyt P450 inhibitors between a metabolism-based chlorsulfuron-resistant biotype SLR31 and a metabolism-based chlorotoluron-resistant biotype WLR2. Malathion was able to increase chlorsulfuron toxicity for the chlorsulfuron-resistant biotype SLR31; however, PBO and tetcyclasis had little, if any, effect (Figs. 1 and 2, Table 4). The results for the chlorotoluron-resistant biotype WLR2 are almost the direct opposite. Malathion had little or no effect on chlorotoluron toxicity (data not shown), while PBO (data not shown) and tetcyclasis (Table 4) increased chlorotoluron toxicity for biotype WLR2. The mechanistic basis for this difference is yet to be determined. However, one likely explanation is that the enzyme(s) metabolizing chlorotoluron in *L. rigidum* can be inhibited by ABT, tetcyclasis, and PBO, but not by malathion, while the enzyme(s) metabolizing chlorsulfuron can be inhibited by malathion but not by the other compounds.

The results of this study suggest that cyt P450 enzymes may be involved in metabolism-based chlorsulfuron resistance in *L. rigidum*. The findings of Burnet *et al.* (24, 25) suggest that cyt P450s may be involved in chlorotoluron and simazine resistance in certain *L. rigidum* biotypes. It is possible that different enzyme systems with different specificity for both herbicides and inhibitors may be involved in each case. However, studies of the isolated enzyme systems are required to test these hypotheses.

ACKNOWLEDGMENTS

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Detoxifying Enzymes in Adults and Nymphs of the German Cockroach: Evidence for Different Microsomal Monooxygenase Systems

STEVEN M. VALLES, SIMON J. YU, AND PHILIP G. KOEHLER

Department of Entomology and Nematology, University of Florida, Gainesville, Florida 32611-0620

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Detoxification enzyme assays were conducted on last-instar nymphs and adult males of the Village Green strain of German cockroach. Activities of glutathione transferases, general esterase, carboxyesterases, and reductases were the same in nymphs and adult males. However, microsomal enzyme assays revealed that aldrin epoxidase, phorate sulfoxidase, methoxyresorufin *O*-demethylase, and biphenyl hydroxylase activities were significantly higher (4- to 15-fold) in the adult male, whereas aniline hydroxylase, ethoxycoumarin *O*-deethylase, *p*-chloro-*N*-methylaniline *N*-demethylase activities and total cytochrome P450 content were not different. Equivalent cytochrome P450 content and disparate enzyme activity toward model substrates in nymphs and adult males suggest that the microsomal monooxygenase systems are different between the two stages. © 1994 Academic Press, Inc.

INTRODUCTION

The German cockroach *Blattella germanica* (L.) is a serious urban pest that has developed resistance to insecticides in the organochlorine, organophosphate, carbamate, and pyrethroid insecticide classes (1). Resistance levels in the German cockroach are typically ascertained by comparing selected lethal dose values of resistant and susceptible strains using the adult male as the test developmental stage (2). Adult females and immature stages generally are not used because female reproductive status influences the level of resistance (2), and nymphs are difficult to stage accurately (3).

Recently, Koehler *et al.* (4) have shown that mixed-sex late-instar German cockroach nymphs (4th–6th instars) were significantly more tolerant to insecticides in the pyrethroid, carbamate, and organophosphate classes than adult males in topical and residual insecticide bioassays. They concluded that stage-dependent differential susceptibility to insecticides may contribute significantly to German cockroach control failures. Moreover, strain/population resistance determinations may be underestimated when using the adult male stage as test subject exclusively. Stage- and gender-dependent differential susceptibility have been documented in the German cockroach by other investigators (5–8). The present studies were carried out to compare microsomal oxidase, hydrolase, glutathione transferase, and reductase activities in last-instar nymph and adult male Village Green German cockroaches.

MATERIALS AND METHODS

Insects. Adults (1 to 2 weeks old) and last-instar nymphs (mid-stadium) of the multi-insecticide-resistant Village Green strain of German cockroach were used in all tests and were obtained from the USDA (Gainesville, FL). The cockroaches were reared as described by Koehler and Patterson (9) at 26°C, 55% RH, and with a 12:12 L:D photoperiod. Village Green cockroaches were subjected to selection pressure since 1990 by periodical exposure to cypermethrin residue on plywood panels placed under watering stations. Cockroaches used in this study were progeny of the selected cockroaches. Nymphs were staged and sexed by the methods of

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Koehler *et al.* (4) and Ross and Cochran (10), respectively.

Chemicals. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP, NADPH, 7-ethoxycoumarin, 7-hydroxycoumarin, *p*-aminophenol, EDTA, bovine serum albumin (BSA), dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), glutathione, *n*-octylamine, α-naphthyl acetate (α-NA), eserine, α-naphthol, acetylthiocholine, juglone, and cytochrome *c* were purchased from Sigma (St. Louis, MO). *p*-Chloraniline, phenylthiourea (PTU), and 3,4-dichloronitrobenzene were purchased from Eastman Kodak (Rochester, NY). Biphenyl, *l*-menthone, and helicin were obtained from Aldrich (Milwaukee, WI). Aniline and glycerol were purchased from Fisher Scientific (Fair Lawn, NJ). All other chemicals were of analytical quality and procured from commercial suppliers.

Preparation of microsomes. Ten to 30 cockroaches, depending upon the assay and microsomal protein required, with their legs, wings (for adults), and head excised, were homogenized for 30 sec in 20 ml of ice-cold 0.1 *M* sodium phosphate buffer, pH 7.5, using a Potter–Elvehjem Teflon pestle and glass mortar. The homogenate was filtered through two layers of cheese cloth, then centrifuged at $10{,}000g_{max}$ for 15 min. The supernatant was filtered through glass wool and further centrifuged at $105{,}000g_{max}$ for 1 hr. The resulting pellet (microsomes) was suspended in buffer specific for each reaction type as indicated. Protein levels were adjusted to 1 mg/ml. The above procedures were all performed at 0–4°C. Protein determinations were made by the method of Bradford (11) using BSA as the standard.

Statistical analysis. Comparisons of mean enzyme activities were accomplished using Student's *t* test. Methoxyresorufin (MR) *O*-demethylase activity and P450 content in different nymphal instars were compared by analysis of variance using proc GLM (12).

Enzyme assays. All enzyme reactions were conducted within linear ranges of protein concentration and incubation time which had been determined in preliminary experiments. At least four experiments, each with duplicate determinations, were conducted for all enzymes assayed.

Microsomal epoxidase activity was measured by the epoxidation of aldrin to dieldrin as described previously (13). Microsomal hydroxylase activity was measured by the hydroxylation of biphenyl to *p*-hydroxybiphenyl as described by Yu and Ing (14). The only modification included the addition of 1 ml 1 *N* HCl prior to the addition of ethyl acetate to improve product recovery.

Aniline hydroxylase activity was determined using a combination of established methods (15–17), which were modified to suit German cockroach enzyme. The 5-ml reaction mixture contained 1 or 2 mg microsomal protein, 0.1 *M* sodium phosphate buffer; an NADPH generating system composed of 1.8 μmol NADP, 18 μmol glucose-6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase, 0.1 *M* KCl, and 10 μmol aniline in 0.1 ml of ethylene glycol monomethyl ether. The reaction mixture was incubated in a water bath with shaking at 32°C in an atmosphere of air for 20 min. Complete reaction mixtures containing equivalent denatured microsomal protein were used as reference blanks. Microsomal protein was denatured by immersion into boiling water for 15 min. The reaction was terminated by the addition of 2 ml of 20% trichloroacetic acid. The mixtures were gently vortexed for 15 sec, then centrifuged for 5 min to separate the precipitated protein. After centrifugation 1 ml of the supernatant was decanted followed by the addition of 0.5 ml of 10% Na_2CO_3 and 1 ml of 2% phenol in 0.2 *M* NaOH. The mixture was allowed to react at room temperature for 1 hr. The resulting indophenol dye was measured spectrophotometrically on a Beckman Model 5260 uv/vis spectrophotometer equipped with a scattered transmission accessory at 620 nm against the corresponding inactive tissue reference.

Quantification was accomplished by reference to a standard curve prepared from known amounts of *p*-aminophenol.

O-Deethylase activity was determined with 7-ethoxycoumarin as substrate (18, 19). The 2.01-ml reaction mixture contained 0.1 *M* Tris–HCl buffer, pH 7.8, 0.075–0.2 mg microsomal protein suspended in 0.1 *M* Tris–HCl buffer, pH 7.8, 0.3 mmol KCl, 2 μmol EDTA; 0.8 μ*M* 7-ethoxycoumarin, and 10 μl of 0.2 m*M* NADPH. The mixture (less NADPH) was incubated in a 30°C water bath for 3 min. The reaction was initiated by the addition of NADPH. Specific activity was determined spectrofluorophotometrically on a Shimadzu Model RF5000U spectrofluorophotometer by the formation of the product, 7-hydroxycoumarin, and was based on the initial reaction rate. The excitation and emission wavelength settings and their respective slit settings were 380 nm (1.5 nm) and 460 nm (3.0 nm). Microsomal *O*-demethylase activity was measured by the *O*-demethylation of methoxyresorufin to resorufin as described by Yu (20). *N*-Demethylase activity was based on the *N*-demethylation of *p*-chloro-*N*-methylaniline (PCMA) to *p*-chloroaniline as described previously (13). Microsomal sulfoxidase activity was determined with phorate as substrate (21).

Cytochrome P450 and cytochrome b_5 contents were determined by the method of Omura and Sato (22). Microsomes were suspended in 0.07 *M* sodium phosphate buffer containing 30% glycerol. Type I spectra (substrate interaction) were determined using *l*-menthone as substrate (23). Type I binding was calculated by dividing the change in absorbance from peak to trough by nmol cytochrome P450. The spectral dissociation constants (K_s) were obtained by double reciprocal plot of spectral changes induced by the addition of increasing concentrations of *l*-menthone to a microsomal suspension (1 mg/ml) (24). The type II difference spectra of cytochrome P450 were determined using *n*-octylamine as the ligand. Percentage high spin was calculated with the constants derived by Jefcoate *et al.* (25) and type II binding was calculated by dividing the change in absorbance from peak to trough by nmol cytochrome P450.

Glutathione transferase activity was measured with 3,4-dichloronitrobenzene (DCNB), 1-chloro-2,4-dinitrobenzene (CDNB), and *p*-nitrophenyl acetate (PNPA) as substrates as described by Yu and Nguyen (26). Enzyme preparation was accomplished as described in the preparation of microsomes section. The $105{,}000g_{max}$ soluble fraction (supernatant) was used as the enzyme source. Homogenization and centrifugation took place in 0.1 *M* Tris–HCl buffer, pH 9.0, for DCNB conjugation, 0.1 *M* sodium phosphate buffer, pH 6.5, for CDNB conjugation, and 0.1 *M* sodium phosphate buffer, pH 7.0, for PNPA conjugation.

General esterase and carboxyesterase activities were measured as described by Yu *et al.* (27) using α-NA as substrate. Ten adult males or last-instar nymphs with legs, wings (adults), and heads excised were homogenized in 20 ml of 0.04 *M* sodium phosphate buffer, pH 7.0. The homogenate was filtered through two layers of cheesecloth, then centrifuged at $1000g_{max}$ for 15 min. The supernatant was used as the enzyme source. To measure carboxyesterase activity, eserine (10^{-4} *M* final concentration) and *p*-hydroxymercury benzoate (10^{-4} *M* final concentration) were added to the reaction mixture to eliminate cholinesterase and arylesterase activities, respectively.

Acetylcholinesterase activity was measured with acetylthiocholine as substrate as described by Ellman *et al.* (28). Fifty adult males or last-instar nymphs were decapitated and the heads homogenized in 10 ml of 0.1 *M* sodium phosphate buffer, pH 8.0. The homogenate was filtered through two layers of cheesecloth, then centrifuged at $1000g_{max}$ for 15 min. The supernatant was used as the enzyme source.

Permethrin esterase was determined using *trans*-permethrin as substrate by the

method of Yu (29). β-Glucosidase activity was measured with helicin as substrate as described previously (30). Quinone reductase was measured using juglone as substrate by the method of Yu (31). Cytochrome *c* reductase activity was determined using the method described previously (21).

RESULTS

Enzyme assays. Preliminary experiments revealed that total cytochrome P450 content (nmol P450/mg protein) was the same in all mixed-sex nymphal instars. Similarly, MR *O*-demethylase activity in ultimate and penultimate instar male and female nymphs and 2nd–6th mixed-sex instar nymphs was not significantly different. Therefore, mixed-sex last-instar nymphs were used as the comparative stage against adult males in all biochemical analyses.

Table 1 shows various detoxifying enzyme activities in adult males and last-instar nymphs. Adults contained an average 0.45 nmol cytochrome P450 per milligram protein which was not statistically different from the nymphs with 0.58 nmol cytochrome P450 per milligram protein. Cytochrome b_5 content (nmol/mg protein) was significantly greater in adult males than in nymphs. Activities of aldrin epoxidase, biphenyl hydroxylase, MR *O*-demethylase, and phorate sulfoxidase were significantly higher in the adult compared to those in the nymph. There was no difference in aniline hydroxylase, ethoxycoumarin *O*-deethylase, or PCMA *N*-demethylase activity between nymphs and adult males. The individual addition of 1.5% BSA, an alternative substrate for proteases, 1 m*M* EDTA, a chelating reagent, 0.1 m*M* DTT, a sulfhydryl group protectant, 10% glycerol, a P450 stabilizer, 1 m*M* PTU, a tyrosinase inhibitor, or 1 m*M* PMSF, a serine protease inhibitor, to the homogenization and suspension buffers did not increase MR *O*-demethylase activity significantly in either nymph or adult male microsomes.

Spectral analysis of cytochrome P450 showed that neither type I nor type II binding was significantly different between adult males and nymphs (Table 2). Percentage high spin cytochrome P450 content was also not significantly different between adults and nymphs. Finally, the absorbance maximum and minimum for type I and type II spectra were identical for both stages.

No significant differences in glutathione transferase activity were observed on a protein basis regardless of substrate (Table 1). However, glutathione transferase activities toward PNPA and CDNB were significantly higher in last-instar nymphs than in adult males on a per insect basis.

α-NA esterase, α-NA carboxyesterase, permethrin esterase, and acetylcholinesterase activities were similar for both developmental stages (Table 1). No differences in juglone reductase or cytochrome *c* reductase activity between the two stages were observed. Helicin glucosidase activity on a protein basis was similar in nymphs and adults, but significantly greater in nymphs on an insect basis.

DISCUSSION

Koehler *et al.* (4) reported that late-stage Village Green nymphs were significantly more tolerant to bendiocarb, cypermethrin, and chlorpyrifos than adult males. Based on detoxification enzyme data utilizing model substrates, it appears that enhanced metabolism may not be responsible for the differential susceptibility reported previously (4). Among the enzymes assayed, nymphs had relatively low microsomal monooxygenase activities compared to adult males; activity was often marginally measurable in nymphs. Additionally, glutathione transferase, hydrolase, and reductase enzyme activities were similar between the two stages. Disparate developmental detoxification enzyme activity within a species is a common and anticipated phenomenon due to changing physiological processes. Turnquist and Brindley (32) showed that cytochrome P450 level and microsomal oxidase activities were low

TABLE 1

Detoxifying Enzyme Activities of Last-Instar Nymphs and Adult Males of the Village Green German Cockroach

Detoxifying enzyme	Enzyme activity					
	nmol/min/mg protein[a]			nmol/min/insect[a]		
	Adult	Nymph	A/N[e]	Adult	Nymph	A/N[e]
Microsomal oxidases						
Cytochrome P450	0.45 ± 0.04[c]	0.58 ± 0.09[c]	0.8	0.16 ± 0.01[d]	0.17 ± 0.02[d]	0.9
Cytochrome b_5	0.32 ± 0.005[c,]*	0.25 ± 0.02[c]	1.3	0.09 ± 0.009[d,]*	0.11 ± 0.006[d]	0.8
Aldrin epoxidation	0.21 ± 0.07[b,]*	0.024 ± 0.01	8.8	0.034 ± 0.01**	0.0030 ± 0.001	11.3
Biphenyl hydroxylation	0.99 ± 0.12**	0.066 ± 0.04	15	0.24 ± 0.09*	0.018 ± 0.01	13.3
Aniline hydroxylation	0.11 ± 0.02	0.029 ± 0.03	3.8	0.037 ± 0.003	0.012 ± 0.01	3.1
MR *O*-demethylation	0.13 ± 0.02**	0.023 ± 0.004	6.5	0.031 ± 0.005**	0.0076 ± 0.001	4.1
EC *O*-deethylation	0.27 ± 0.08	0.10 ± 0.03	2.7	0.065 ± 0.02	0.028 ± 0.01	2.3
PCMA *N*-demethylation	1.22 ± 0.17	1.07 ± 0.21	1.1	0.32 ± 0.06	0.41 ± 0.1	0.8
Phorate Sulfoxidation	0.48 ± 0.14*	0.053 ± 0.01	9.1	0.12 ± 0.04*	0.018 ± 0.005	6.7
Glutathione transferases						
DCNB conjugation	36.97 ± 2.96	24.65 ± 3.75	1.5	59.0 ± 6.58	56.5 ± 9.1	1.0
CDNB conjugation	2174.66 ± 111.67	2144.83 ± 86.43	1.0	2597.69 ± 221.35**	4524.67 ± 146.79	0.6
PNPA conjugation	247.28 ± 5.39	198.61 ± 16.91	1.3	343.64 ± 22.62*	457.03 ± 32.8	0.8
Hydrolases						
α-NA esterase	237.08 ± 18.37	217.45 ± 36.47	1.1	662.0 ± 26.0	850.0 ± 91.0	0.8
α-NA carboxyesterase	89.12 ± 5.47	88.07 ± 8.63	1.0	286.0 ± 7.0	350.0 ± 23.0	0.8
Acetylcholinesterase	47.21 ± 9.87	27.81 ± 2.69	1.7	9.5 ± 2.22	7.05 ± 0.82	1.4
Permethrin esterase	1.34 ± 0.04	1.21 ± 0.24	1.1	1.74 ± 0.12	3.08 ± 0.55	0.6
Helicin β-glucosidase	1.96 ± 0.19	1.97 ± 0.14	1.0	3.27 ± 0.21**	5.14 ± 0.33	0.6
Reductases						
Cytochrome *c* reductase	24.93 ± 6.15	23.03 ± 4.8	1.1	9.56 ± 2.47	11.82 ± 3.11	0.8
Juglone reductase	24.34 ± 4.49	29.0 ± 7.73	0.8	8.02 ± 0.52	10.65 ± 2.52	0.8

[a] Mean ± SE.

[b] Value significantly different from the nymphal stage. $*P < 0.05$; $**P < 0.01$.

[c] nmol/mg protein.

[d] nmol/insect.

[e] Adult male enzyme activity/nymph enzyme activity.

TABLE 2

Type I (l-menthone) and Type II (n-octylamine) Spectral Characteristics of Microsomes from Last-Instar Nymphs and Adult Males of the Village Green German Cockroach

Spectral characteristic	Developmental stage	
	Adult	Nymph
Type II binding[a]	0.060 ± 0.005	0.055 ± 0.001
% High spin P450[b]	12.1 ± 2.1	12.9 ± 1.3
Type II λ_{max}	432	432
Type II λ_{min}	408	408
Type I binding[c]	0.030 ± 0.003	0.026 ± 0.0015
Type I K_s (m*M*)	10.2 ± 1.63	9.69 ± 3.91
Type I λ_{max}	390	390
Type I λ_{min}	420	420

[a] Type II binding = Δ absorbance *n*-octylamine spectrum/nmol P450/ml.

[b] Calculated with the constants derived by Jefcoate *et al.* (25).

[c] Type I binding = Δ absorbance *l*-menthone spectrum/nmol P450/ml.

in newly eclosed adult male and female American cockroaches [*Periplaneta americana* (L.)] gradually rising to a peak at about 90–100 days, then declining to low levels at 130–140 days after eclosion. Benke *et al.* (33) reported that aldrin epoxidase activity varied between instars and within stadia of the Madagascar cockroach, *Gromphadorhina portentosa* (L.). Numerous other examples of stage-dependent cytochrome P450 monooxygenase activity have been reported in insects (34, 35).

Several hypotheses may be presented to explain the discrepancies in cytochrome P450 content and activity, with the assumption that multiple cytochrome P450 isozymes are present, for example, as described in the house fly (36, 37). Nymphs and adults may contain the same complement of cytochrome P450 isozymes, but many of the forms in the nymph may be inactive. Specifically, some forms could require post-translational modification (e.g., proteolytic cleavage or covalent modification) to activate them. This could explain equivalent P450 content and lower enzyme activity in the nymph. Alternatively, low nymphal microsomal monooxygenase activity could be due to an inhibitor in the nymph which is not present in the adult male stage. However, experimental evidence indicates that this is probably not the case. Various reagents used to protect or stabilize the microsomal monooxygenases added individually during homogenization (glycerol, EDTA, DTT, PMSF, PTU, and BSA) did not significantly improve enzyme activity. Also, although the majority of the nymphal enzymes exhibited low activity, PCMA *N*-demethylation was equally high in both stages suggesting that the technique for microsomal preparation was sound. Moreover, cytochrome P420 content (results not shown) was low in both nymphs and adults, indicating that the P450 isozymes were not being degraded by endogenous enzymes during microsomal preparation. Finally, adult male microsomes did not lose their MR *O*-demethylase activity when combined with nymph microsomes, further indicating that an inhibitor was probably not present in the nymphal stage.

Finally, nymphs and adult males may possess dissimilar complements of cytochrome P450 isozymes. Obviously, both stages contain equal genetic makeup, but because of different physiological requirements, each developmental stage may express/repress various cytochrome P450 isozymes selectively. Developmental regulation of cytochrome P450 isozymes has been demonstrated in the house fly. Wheelock *et al.* (38) showed that cytochrome $P450_{lpr}$, an isozyme isolated from the pyrethroid-resistant LPR house fly strain (39), was present in the house fly adult, barely detectable in the pupae, and not detectable in the larvae. Additionally, Feyereisen and Farnsworth (40) found that cytochrome P450 monooxygenase enzyme activity was specific for each developmental stage in the cockroach, *Diploptera punctata* (Eschscholtz). Last, numerous xenobiotics selectively induce the production of cytochrome P450 isozymes (41, 42).

It appears that cytochrome P450 enzymes may be developmentally regulated in the German cockroach. Nymphs and adult

males contained similar total cytochrome P450, while cytochrome P450 monooxygenase activities were different. Disparate cytochrome P450 monooxygenase activities with similar total cytochrome P450 content were also observed in the Saint Joseph German cockroach strain (Valles and Yu, unpublished results) suggesting that this phenomenon may be inherent to the species. Work is currently under way to further characterize the developmental expression of cytochrome P450 isozymes in the German cockroach.

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In Vitro Metabolism of Parathion in Susceptible and Parathion-Resistant Strains of the Greenbug, *Schizaphis graminum* (Rondani) (Homoptera: Aphididae)

MIDORI ONO, JONATHAN S. RICHMAN, AND BLAIR D. SIEGFRIED[1]

Department of Entomology, 202 Plant Industry Building, University of Nebraska, Lincoln, Nebraska 68503

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Results of *in vitro* metabolism studies in a susceptible and two parathion-resistant greenbug strains indicate that hydrolytic enzymes are active toward paraoxon, but not parathion, and are partially responsible for resistance. The resistance does not involve reduced levels of oxidative metabolism and activation of parathion since NADPH-dependent microsomal formation of paraoxon in resistant and susceptible strains was similar. Paraoxon is a potent inhibitor of greenbug esterases, and the resistance mechanism apparently involves rapid and tight binding of enzyme and insecticide that prevents the insecticide from reaching its target site. Native polyacrylamide gel electrophoresis also supported this view since staining of enhanced bands associated with resistance was inhibited by incubation with paraoxon. The resistant strains apparently produce more of the isozymes that are inhibited by paraoxon and are able to sequester a greater number of paraoxon molecules than susceptible greenbugs. © 1994 Academic Press, Inc.

INTRODUCTION

Greenbugs, *Schizaphis graminum* (Rondani) (Homoptera: Aphididae), are pests of small grains and sorghum throughout the midwestern United States. Organophosphate (OP)[2] insecticides, such as parathion, have been widely used to control greenbugs. However, by the mid-1970s widespread resistance of the greenbug to the systemic organophosphate, disulfoton, resulted in increased application rates and an eventual rotation to other insecticides (1, 2). Although the presence of resistance in greenbugs resulted in control failures and economic loss, the development of various resistant sorghum cultivars circumvented the resistance problem (1, 2), and the study of insecticide resistance in greenbugs was largely discontinued. Insecticides are still frequently used to control greenbugs, however, especially after the development of new biotypes that are capable of overcoming a resistant cultivar's defenses. Recent reports of control failures from several areas of the Midwest (3) have resulted in renewed interest in greenbug resistance to insecticides.

The mechanism of resistance in greenbug strains collected from areas of control failures appears to involve esterase-mediated insecticide detoxification (4, 5). Resistant greenbugs display enhanced nonspecific esterase activity, although different greenbug strains with different patterns of enhanced esterase bands have been observed (6). These resistant strains exhibit 20- to 30-fold resistance to parathion (4, 5), and *in vivo* metabolism of [^{14}C]parathion by resistant and susceptible strains indicates that resistance is correlated to reduced levels of paraoxon. It was proposed that reduced paraoxon was the result of higher levels of hydrolytic enzymes active toward paraoxon rather than reduced rates of oxidative desulfuration. However, the role of oxidative enzymes in the resistance could not be completely excluded, and differences in activity of hydrolytic enzymes toward insecticide substrates have not been conclusively demonstrated.

The following investigation was con-

[1] To whom correspondence should be addressed.

[2] Abbreviations used: OP, organophosphate; TLC thin-layer chromatography; CPBA, *m*-chloroperoxybenzoic acid.

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ducted to determine the relative roles of hydrolytic and oxidative metabolism in the resistance of greenbugs to organophosphate insecticides. *In vitro* metabolism studies were performed to assess the relative roles of the two enzyme systems with respect to metabolism of and resulting resistance to parathion.

MATERIALS AND METHODS

Greenbug strains. The two parathion-resistant greenbug strains were established from field collections in areas of control failure with OP insecticides. A colony collected from western Kansas was provided by Dr. Bill Black, Kansas State University, and was established at the University of Nebraska in January 1990. This strain is referred to as the Type I resistant strain based on the pattern of esterase isozymes observed by gel electrophoresis (4, 6). The other resistant strain was collected in Sherman County, Texas, and a colony was established at the University of Nebraska in October 1991 after a colony was provided by Ms. Roxanne Shufran, Kansas State University. This strain is referred to as Type II again based on esterase banding pattern (5, 6). The susceptible greenbugs were collected from Lancaster County, Nebraska, in August 1990. Preliminary bioassays indicated susceptibility to parathion and disulfoton. All colonies were maintained on sorghum seedlings (Funks G-522A) at 26°C and a 16:8 (L:D) photoperiod.

Chemicals. Technical grade parathion (99.9% active ingredient) was provided by Dr. Tom Holtzer, Colorado State University. [^{14}C]Parathion (ring-labeled, 34.2 mCi/mmol) was a gift from Cheminova Agro (Lemvig, Denmark). Radioactive parathion was purified from the sample by thin-layer chromatography (TLC) on 0.25-mm LK5-DF silica gel plates (Whatman, Clifton, NJ). Plates were developed first in 100% toluene followed by chloroform:hexane:ethanol (88:10:2 by volume). An X-ray film (Kodak Diagnostic X-ray film) was exposed to the TLC plate for 3 hr and radioactive bands were identified by autoradiography. The radiolabeled parathion was identified by cochromatography with cold parathion standard, scraped from the plate, and eluted into 100% ethanol. [^{14}C]Paraoxon was prepared by incubation of parathion with *m*-chloroperoxybenzoic acid (CPBA) in diethyl ether (0.59 μmol parathion: 1.77 μmol CPBA) for 30 min. [^{14}C]Paraoxon was purified from the resulting mixture by TLC and autoradiography as described previously. Cold paraoxon (95% active ingredient) and *p*-nitrophenol were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were of reagent grade or better.

In vitro metabolism. Aphids were homogenized in 0.02 *M* sodium phosphate buffer (pH 7.0) with a Teflon glass Potter–Elvehjem homogenizer. The homogenate was centrifuged at 10,000*g* for 20 min in a microcentrifuge, and protein concentration of the supernatant was determined by the method of Bradford (7). The protein concentration was adjusted to 10 mg/ml. Each reaction mixture consisted of 1 mg protein in 0.02 *M* sodium phosphate buffer (pH 7.0) and 0.04 μCi parathion or paraoxon introduced in 5 μl ethanol for a final volume of 1 ml. The reaction was initiated by the addition of paraoxon, and samples were incubated at 30°C with shaking. After incubation for varying periods of time or immediately after the addition of substrate to the enzyme, the reaction was stopped by the addition of 2 ml ethyl acetate. A combined ethyl acetate extract (2 × 2 ml) of the incubation was concentrated under N_2 and applied to a TLC plate which was developed in the same solvents described for [^{14}C]parathion purification. Each metabolite and unmetabolized parathion or paraoxon were identified by cochromatography with cold standards, scraped into a scintillation vial, and radioactivity was determined by liquid scintillation counting.

For microsomal metabolism experiments, greenbugs were homogenized in 1.0

ml of 50 m*M* sucrose in 0.1 *M* sodium phosphate buffer (pH 7.4), and the homogenate was centrifuged at 10,000*g* for 20 min. The supernatant was transferred to an ultracentrifuge tube and centrifuged further at 100,000*g* for 1 hr in a Beckman (Palo Alto, CA) TL-100 ultracentrifuge. The pellet was resuspended in 550 μl of the same buffer by homogenization with a Teflon glass Potter–Elvehjem homogenizer. Each reaction mixture contained 1 μmol of NADPH, 0.09 μCi [^{14}C]parathion introduced in 5 μl ethanol and 250 μl of the microsome preparation. Control incubations lacked NADPH. The total volume was adjusted to 1 ml with 0.1 *M* sodium phosphate buffer (pH 7.4). The reaction was started by the addition of NADPH, and all tubes were incubated at 30°C in a shaking water bath for 1.5 hr. The reaction was stopped by the addition of 2 ml of ethyl acetate, and extraction and analysis of metabolites were performed as previously described.

Inhibition study. Enzyme preparations were made as described above with 0.02 *M* sodium phosphate buffer (pH 7.0) with 1% Triton X-100 and protein concentration was adjusted to 1 mg/ml. Each enzyme preparation (0.1 mg protein) was incubated with varying concentrations of paraoxon in sodium phosphate buffer (0.02 *M*, pH 7.0) in a total of 2 ml solution. The enzyme preparation was assayed for activity toward *p*-nitrophenyl acetate 2 min after addition of inhibitor. Each reaction mixture (1 ml in total) contained 50 μl inhibited enzyme in 0.02 *M* sodium phosphate buffer (pH 7.0) and 0.5 m*M* *p*-nitrophenyl acetate in acetone. The reaction was started by the addition of *p*-nitrophenyl acetate. Absorbance at 400 nm was read every 6 sec for 2 min and the slope was calculated from the change in absorbance.

Esterase isozymes from resistant and susceptible greenbugs were separated by discontinuous polyacrylamide electrophoresis under native conditions. Two identical gels were run simultaneously. After completion, one gel was incubated in sodium phosphate buffer (0.02 *M*, pH 7.0) and the other in 0.6 m*M* paraoxon in the same buffer for 30 min at 30°C. Both gels were then stained for esterase activity as described previously (8).

Statistical analyses. Each metabolite was expressed as percentage of the total radioactivity recovered and transformed by the arcsine square root transformation (9). For each metabolism study, resistant strains were compared with the susceptible strain by a two sample *t* test (10).

RESULTS

In vitro metabolism. Studies with the 10,000*g* supernatant from greenbug homogenates and [^{14}C]parathion as substrate showed no significant metabolite formation, since levels of potential metabolites detected were not different from those observed in control incubations lacking enzyme preparation (data not shown). However, when paraoxon was used as substrate, significant metabolism was detected (Table 1), based on formation of *p*-nitrophenol. The Type II and Type I strains exhibited significantly lower levels of paraoxon and higher levels of *p*-nitrophenol relative to the susceptible strain. Type II consistently displayed lower levels of paraoxon and higher levels of *p*-nitrophenol than Type I. All three strains exhibited decreasing paraoxon and increasing *p*-nitrophenol as incubation time increased (Table 1), although significant levels of *p*-nitrophenol were formed immediately after addition of paraoxon to the enzyme (Fig. 1). The rapid formation of *p*-nitrophenol followed by much slower rates of formation indicate that the interaction of greenbug esterases with paraoxon may be different from true detoxification by hydrolysis. The initial binding of esterase to paraoxon may be indicative of phosphorylation of the enzyme and subsequent inhibition.

In vitro metabolism with microsomal preparations. In vitro metabolism experiments with microsomal preparations indicated that the NADPH-dependent forma-

TABLE 1
Percentage Recovery of Radioactivity in in Vitro Metabolism of Paraoxon

Time (min)	Metabolite	Strain (% Recovery ± SEM)[a]		
		Susceptible	Type I	Type II
0	Paraoxon	98.2 ± 0.1	97.9 ± 0.1*	97.1 ± 0.3*
10	Paraoxon	98.3 ± 0.1	97.9 ± 0.1*	96.9 ± 0.4*
30	Paraoxon	97.8 ± 0.2	97.3 ± 0.2*	96.3 ± 0.4*
60	Paraoxon	97.1 ± 0.2	96.7 ± 0.1	95.8 ± 0.3*
120	Paraoxon	96.5 ± 0.4	96.5 ± 0.1	95.0 ± 0.4*
0	*p*-Nitrophenol	1.49 ± 0.04	1.84 ± 0.02*	2.73 ± 0.28*
10	*p*-Nitrophenol	1.45 ± 0.11	1.82 ± 0.12*	2.91 ± 0.38*
30	*p*-Nitrophenol	1.81 ± 0.12	2.31 ± 0.13*	3.34 ± 0.43*
60	*p*-Nitrophenol	2.41 ± 0.20	2.91 ± 0.15	3.88 ± 0.31*
120	*p*-Nitrophenol	2.90 ± 0.39	3.00 ± 0.12	4.49 ± 0.38*
0	Origin	0.28 ± 0.01	0.29 ± 0.05	0.21 ± 0.01
10	Origin	0.28 ± 0.01	0.28 ± 0.03	0.25 ± 0.02
30	Origin	0.38 ± 0.04	0.36 ± 0.03	0.33 ± 0.02
60	Origin	0.45 ± 0.07	0.40 ± 0.04	0.35 ± 0.03
120	Origin	0.58 ± 0.04	0.53 ± 0.02	0.49 ± 0.05

[a] Each value represents the mean of at least four determinations.
* Significantly different from the susceptible strain ($P < 0.05$; two-sample t test).

tion of paraoxon from parathion was slightly higher in the resistant strains. The formation of paraoxon from parathion was not observed in the absence of NADPH. Specific activities of paraoxon-forming enzymes were 0.97 ± 0.06, 1.53 ± 0.14, and 1.63 ± 0.25 pmol/min/mg protein for susceptible, Type I, and Type II strains, respectively (assay replicated four times for each strain). These results indicate that the resistance mechanism does not involve reduced levels of cytochrome P450-dependent formation of paraoxon from parathion. However, it is not clear if increased oxidative activity is involved in the resistance since increased activity would result in higher rates of activation which is not supported by *in vivo* metabolism experiments (4, 5). In addition, previous examination of microsomal monooxygenase activity toward model substrates indicated no differences among the same resistant and susceptible strains (4, 5).

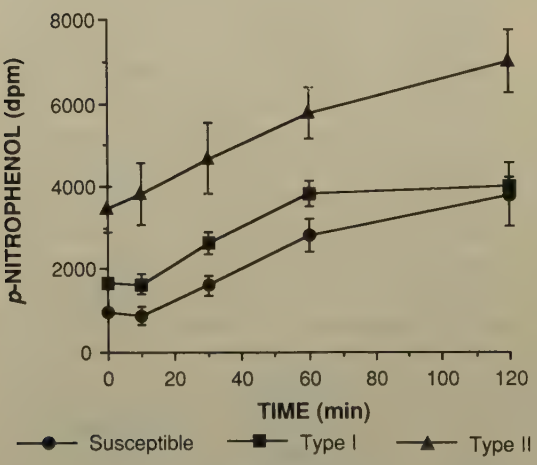

FIG. 1. *In vitro formation of p-nitrophenol from paraoxon using 10,000g supernatant from resistant and susceptible greenbugs. Each point represents the mean of at least four experiments. Bars indicate standard error of the mean.*

Inhibition of esterase activity. Figure 2 represents the inhibition of *p*-nitrophenyl acetate hydrolysis after enzyme preparations were incubated with varying concentrations of paraoxon. For all three strains, esterase activity was strongly inhibited by paraoxon. The complexity of the inhibition curves is probably due to the multiple esterase isozymes present in the prepara-

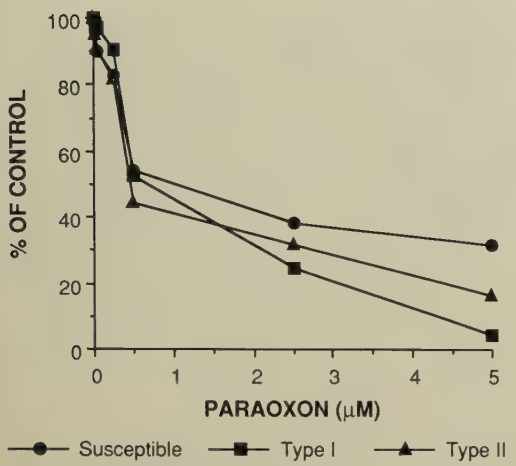

FIG. 2. *Inhibition of p-nitrophenyl acetate hydrolysis by paraoxon from 10,000g supernatant of resistant and susceptible greenbug homogenates. Each point represents the mean of three experiments with three determinations per experiment.*

tions. However, these data support the conclusion from the *in vitro* metabolism studies that the esterase isozymes are tightly bound to paraoxon, resulting in enzyme inhibition. The inhibitory action of paraoxon was further tested by native gel electrophoresis. Polyacrylamide electrophoresis gels under native conditions stained with α-naphthyl acetate with and without inhibition by paraoxon are shown in Fig. 3. After inhibition by paraoxon (3×10^{-7} *M* paraoxon; 0.5 mg protein per lane) enhanced bands in Type I and Type II as

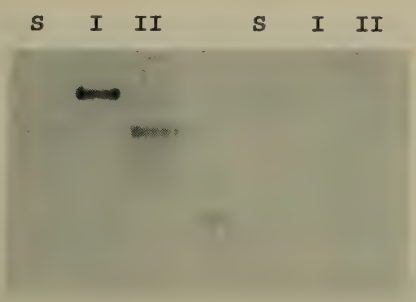

FIG. 3. *Inhibition of general esterase isozymes separated by native polyacrylamide gel electrophoresis from Type I, Type II, and susceptible greenbug strains. The gels incubated with buffer (left) and paraoxon (right), showing inhibition of certain isozymes by paraoxon.*

well as the corresponding bands in the susceptible greenbugs disappeared, supporting inhibition of individual esterase isozymes.

DISCUSSION

Results from *in vitro* metabolism and inhibition studies indicate that hydrolyic enzymes are involved in greenbug resistance to parathion, although the mechanism of resistance appears to involve more of a response to inhibition rather than true catalytic hydrolysis. Quantification of metabolites from *in vivo* metabolism studies (4, 5) indicated that metabolic enzymes are at least partially responsible for resistance based on reduced rates of paraoxon formation in both resistant strains. However, it was unclear whether reduced paraoxon formation was the result of reduced rates of bioactivation of parathion by cytochrome P450-dependent microsomal monooxygenases or increased hydrolytic metabolism of paraoxon. More conclusive evidence for increased hydrolytic metabolism of paraoxon as it relates to resistance is seen in the results of *in vitro* metabolism experiments. The actual substrate of these enzymes appears to be paraoxon, the oxidatively desulfurated and active form of parathion. Because other biologically functional systems and factors were eliminated by the *in vitro* conditions of the assay, the bioactivation of parathion did not take place and, therefore, *in vitro* metabolism of parathion was not detected. Similar results have been reported for *Heliothis virescens* in which hydrolysis was not detected when methyl parathion was used as substrate in contrast to methyl paraoxon, which was metabolized by hydrolytic enzymes (11). Reduced levels of cytochrome P450-dependent microsomal monooxygenases are apparently not responsible for differences in the *in vivo* rates of paraoxon formation since NADPH-dependent microsomal metabolism of parathion was at least as high or higher in the resistant strains. In combination, the results of *in vitro* metabolism experiments in-

dicate that esterase-mediated metabolism of paraoxon is at least partially responsible for resistance in both Type I and Type II strains.

Although it is apparent that greenbug esterases contribute to resistance, the action of these esterases appears to be different from true enzymatic hydrolysis because product formation (i.e., formation of *p*-nitrophenol) was almost complete immediately upon addition of enzyme to substrate. Such results indicate that paraoxon is a better inhibitor than substrate, and that the mechanism of resistance involves enzyme inhibition and sequestration of the toxin rather than detoxification by hydrolysis. Similar resistance mechanisms have been proposed for a number of organophosphate-resistant insects (8, 12, 13). Because the resistant strains possess more of the total esterase, they are capable of binding more of the toxic molecules than the susceptible strain. Since *p*-nitrophenol was formed almost immediately after addition of paraoxon to the enzyme preparation, it appears that initial affinity of the enzyme and insecticide is high and that phosphorylation of the enzyme is rapid. The phosphorylated enzyme apparently is a stable complex resulting in enzyme inhibition.

Although the involvement of nonspecific esterases in greenbug resistance has been established, it is still uncertain if the elevation of these enzymes is the result of overproduction of an existing isozyme or if a mutant isozyme is responsible for increased total esterase in the resistant strains. Continued clarification of the role of these enzymes in the resistance mechanism will lead to a better understanding of the factors responsible for resistance development and ultimately to resistance management programs based on a thorough understanding of the resistance mechanism.

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Potentiation of Carbaryl Toxicity to the Hybrid Red-Legged Partridge Following Exposure to Malathion

GAIL JOHNSTON,* COLIN H. WALKER,* AND ALISTAIR DAWSON†

*Department of Biochemistry and Physiology, School of Animal and Microbial Sciences, AMS Building, University of Reading, Whiteknights, PO Box 228, Reading, Berkshire, RG6 2AJ, United Kingdom; and †Institute of Terrestrial Ecology, Monks Wood, Abbots Ripton, Huntingdon, Cambridgeshire, PE17 2LS, United Kingdom

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Pretreatment of hybrid red-legged partridges (*Alectoris rufa* cross) with a single oral dose of 167 mg/kg malathion resulted in a marked potentiation of the toxicity of the carbamate insecticide, carbaryl, given 1 hr after malathion. Oral administration of 200 mg/kg carbaryl was lethal to 4 out of 12 malathion-pretreated birds, and a further 6 showed symptoms of cholinesterase poisoning. Birds given either malathion or carbaryl alone showed no visible signs of toxicity. The inhibition of serum butyrylcholinesterase activity was found to be high (75–91%) in both corn oil- and malathion-pretreated birds at 1 and 3 hr after dosing with carbaryl, with no significant difference between them. However, malathion and carbaryl given in combination produced an 88% depression of brain acetylcholinesterase activity compared to a 56% depression in corn oil-pretreated birds given carbaryl; this difference was highly significant. Malathion alone produced a small nonsignificant 5% elevation of activity. Concentrations of carbaryl in the serum and brain were found to be 2- and 7.5-fold greater, respectively, in birds pretreated with malathion compared to those given corn oil. *In vitro* metabolism of [^{14}C]carbaryl in the presence of NADPH from control birds resulted in the formation of two metabolites (46% of the carbaryl was metabolized), whereas metabolism of carbaryl by microsomes from birds pretreated with malathion was substantially less: only 3% of the carbaryl was metabolized. The inactivation of cytochrome P450 by reactive sulfur liberated during the oxidative desulfuration of malathion is thought to be responsible for these differences in carbaryl metabolism. The potentiation of carbaryl toxicity by malathion was attributed to an inhibition of metabolic detoxication, due largely or entirely to the inhibition of certain forms of cytochrome P450. Other types of pesticides metabolized by cytochrome P450s, such as pyrethroids and organophosphorus insecticides not requiring activation for toxicity, may, like carbaryl, be potentiated following inhibition. © 1994 Academic Press, Inc.

INTRODUCTION

Animals are frequently exposed to complex mixtures of chemical pollutants in the field, and there has been much debate about the toxicological consequence of such exposures. In the marine environment, fish, mammals, and birds have been found to contain a variety of polychlorinated biphenyls and/or polycyclic aromatic hydrocarbons (1). Birds and mammals inhabiting agricultural land may encounter a number of pesticides, either simultaneously or consecutively. The use of combinations of pesticides in tank mixes or seed dressings raises the possibility that simultaneous exposure to substantial quantities of two or more pesticides may occur.

Usually, the toxicity of two or more chemicals is approximately additive, i.e., the toxicity of a mixture approximates to the summation of the toxicities of the individual components. Sometimes, however, a potentiation of toxicity can occur, in which the toxicity of a mixture is substantially greater than the summation of the toxicities of the individual components. The difficulty lies in discovering whether such interactions occur in the field.

With pesticides, it is not possible to test the toxicity of more than a very small proportion of the total number of combinations of chemicals that animals may be exposed to on agricultural land, and then only a few species are available for testing purposes.

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The basic problem lies in identifying those combinations of chemicals that may give rise to a potentiation of toxicity. Hence, there is a case for adopting a mechanistic approach, whereby certain combinations of chemicals may be identified for which there is a risk that a potentiation of toxicity will occur as a consequence of recognized mechanisms of interaction.

At the toxicokinetic[1] level there are two major ways in which one chemical may cause another to become more toxic; (i) when one chemical enhances the metabolic activation of another, (ii) when one chemical inhibits the detoxication of another. Potentiation of toxicity by the first mechanism has already been demonstrated in the hybrid red-legged partridge (*Alectoris rufa* cross) (2). Ergosterol biosynthesis inhibiting fungicides such as prochloraz can strongly induce hepatic microsomal monooxygenases (HMOs), which have cytochrome P450 as the active site (3–6). Birds which have been so induced become more susceptible to the toxic action of organophosphorus insecticides (OPs), such as malathion and dimethoate, which require metabolic activation by HMOs (2, 7). For example, partridges which were dosed with 90 or 180 mg/kg prochloraz showed substantially greater inhibition of serum butyrylcholinesterase (BuChE) activity after dosing with malathion than did control birds. This effect was shown at different dose levels of malathion. Similar effects were also shown in the pigeon (*Columba livia*).

The present study was designed to investigate the second mechanism of interaction mentioned above, using a combination of an OP insecticide (malathion) with a carbamate insecticide (carbaryl), again in the partridge. Both OP and carbamate pesticides are widely used in agriculture, and there have been cases in which mammals and birds have been lethally poisoned by pesticides of both groups on agricultural land (8–10). Also, there are situations in which exposure to both types of compounds may occur in the field: for example, when two different types of pesticide are used on the same area of land (such as an OP insecticide which has been sprayed in a field already treated with a granular formulation or a carbamate molluscicide, or where a bird moves between different fields which have been treated with different chemicals). This type of combination is of interest because OPs may inhibit the enzymatic detoxication of carbamates, and therefore there is a possibility of potentiation.

[1] The term "toxicokinetics" encompasses the process of uptake, distribution, storage, metabolism, and excretion, which determine the extent to which toxic molecules reach their sites of action.

MATERIALS AND METHODS

Birds and Treatments

Sexually mature male hybrid red-legged partridges were obtained from Lincolnshire Pheasantries, Boston, Lincolnshire, United Kingdom. The hybrid is a cross between the red-legged partridge (*A. rufa*) and two other species of *Alectoris:* the rock partridge (*A. graeca*) and the chukar partridge (*A. chukar*). Birds were housed outdoors communally in aviaries and provided with turkey starter crumbs (Heygate and Sons, Northampton, UK) and water *ad libitum*.

Birds were divided into four different treatment groups as shown in Fig. 1. Corn oil (0.4 ml/kg) was given to two of these groups by oral intubation, and malathion (167 mg/kg) in corn oil (0.4 ml/kg) was given to the other two groups. After 1 hr, one of the corn oil-pretreated and one of the malathion-pretreated groups were given a single oral dose of 200 mg/kg carbaryl, which was administered orally in gelatin capsules due to the low solubility of this compound in oil. Blood samples were taken immediately prior to dosing with either corn oil or malathion and at 2 and 4 hr after dosing with oil or malathion (1 and 3 hr after dosing with carbaryl). In addition, blood samples were taken from the group dosed with malathion alone after 1 hr. Birds were sacrificed after the same time interval, that is, 4

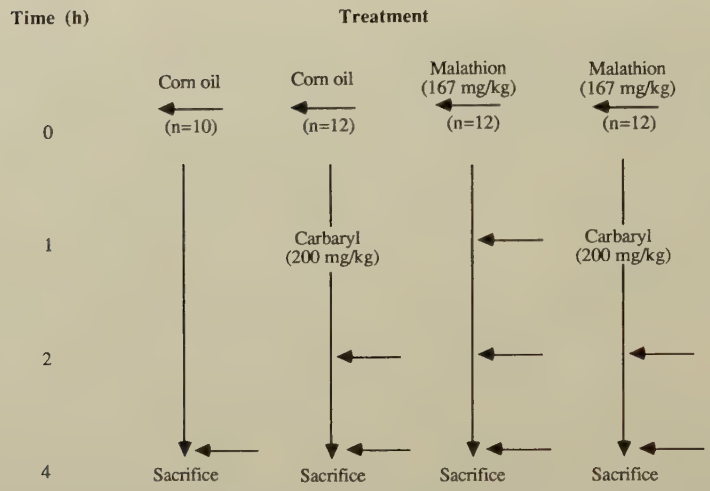

FIG. 1. *Treatment groups showing times of administration of corn oil, malathion, and carbaryl. Arrows denote blood sampling times.*

hr after dosing with corn oil or malathion or 3 hr after dosing with carbaryl.

Chemicals

Analytical grade malathion (*O,O*-dimethyl *S*-[1,2-bis(ethoxycarbonyl)ethyl]phosphorodithioate), 98–99% purity, was obtained from Greyhound Chromatography and Allied Chemicals (Birkenhead, U.K.). Carbaryl (1-naphthyl-*N*-methylcarbamate, 99% purity) was a gift from Embetec Crop Protection, Harrogate, Yorkshire, U.K.). Methyl-labeled [^{14}C]carbaryl (57 mCi/mmol) was obtained from Amersham International plc (Buckinghamshire, U.K.). Unlabeled carbaryl with a purity of 99.4% obtained from the National Physical Laboratory (Teddington, Middlesex, U.K.) was added to this to produce [^{14}C]carbaryl or lower specific activity (0.1 mCi/mmol) for microsomal incubations.

Serum BuChE and Brain Acetylcholinesterase (AChE) Assay

Serum was separated by centrifugation of blood at 13,000 rpm for 10 min and was assayed within 2 hr for BuChE activity. Samples were kept at 4°C before and after centrifugation.

The BuChE activity of serum was determined colorimetrically by the method of Ellman *et al.* (11). Butyrylthiocholine iodide was used as the substrate, and released thiocholine was detected by reaction with 5,5′-dithio-bis(2-nitrobenzoic acid) and measured using a Gilford 240 recording spectrophotometer fitted with a thermostatic cuvette holder. Absorbance was measured at 412 nm and the reaction carried out at 37°C. BuChE activity was calculated using the extinction coefficient of the 5-thio-2-nitrobenzoate ion and was expressed in micromoles per minute per milliliter serum.

Whole brains were divided into quarters, and a portion of each quarter was homogenized in Tris–HCl buffer (25 m*M*) containing 0.1% Triton X-100 to a concentration of 0.06 g brain/ml using a Potter–Elvehjem glass–Teflon homogenizer. AChE activity was assayed within 2 hr of death in the same manner as described for serum, except that acetylthiocholine iodide was used as the substrate.

Analysis for Carbaryl in Serum and Brain

The amount of carbaryl present in serum 1 and 3 hr after carbaryl administration was determined by high-performance liquid

chromatography (HPLC). Serum from control birds was also analyzed. Prior to analysis, carbaryl and metabolites were extracted from serum samples (0.05–1.0 ml) by whirlimixing with 0.5 ml methanol (modified from the method described by Lord *et al.* (12). Samples were then centrifuged at 2000 rpm for 5 min in an MSE Centaur 2 benchtop centrifuge, and the top layer was removed. A further two methanol extractions were carried out, and extracts were dried by the addition of anhydrous sodium sulphate. In order to clean up the extracted carbaryl and its metabolites, extracts were blown down with nitrogen and transferred to thin-layer chromatography (TLC) plates using methanol. TLC was performed on plates which contained a fluorescent indicator (silica gel 60 F254 20 × 20 cm, Merck). A carbaryl standard was applied as a marker. Plates were developed using dichloromethane as a solvent. Carbaryl was located by examination of plates under ultraviolet radiation (254 nm), and areas containing carbaryl were individually scraped and eluted from the plates with dried HPLC-grade methanol. Samples were then partially blown down with nitrogen, and where necessary, were filtered using a disposable filter assembly (Gelman Acro LC3A, 0.45 μm). HPLC was carried out with an ACS ternary solvent system 353 pump, a Rheodyne 7125 injection valve (fitted with a 20-μl loop), and a Knauer 11460 variable wavelength detector. Reversed-phase HPLC of carbaryl was performed using a 250 × 4.6-mm stainless-steel column (Hichrom Ltd., Reading) packed with Spherisorb S5ODS1 silica. Chromatographic separation was achieved with an acetonitrile–water mixture (60:40, v/v) to which 2% methanol was added, and a flow rate of 2 ml/min. Carbaryl was quantified by comparison with standard samples of known concentrations.

Carbaryl present in the brain of birds 3 hr after carbaryl administration was also determined by HPLC. Whole brains or portions of brain taken after division of the whole brain into quarters were homogenized in methanol (0.5 g/ml) using a Potter–Elvehjem glass–Teflon homogenizer. Carbaryl was extracted using methanol as described for serum, and HPLC analysis was carried out in the same manner as that given for serum.

Metabolism of [^{14}C]Carbaryl in Vitro

A comparison of the metabolism of [^{14}C]carbaryl *in vitro* by microsomes prepared from either control or malathion-treated birds by the method of Chipman *et al.* (13) was carried out. Each assay consisted of 4.7 or 4.8 ml phosphate buffer, pH 7.4 (55 m*M* sodium dihydrogen orthophosphate, 13.4 m*M* KCl, and 4.8 m*M* nicotinamide), and 0.2 ml microsomal suspension (1 g liver/ml). An NADPH-generating system (0.1 ml) consisting of 21 μmol glucose 6-phosphate, 4.6 μmol NADP, and 1.6 U of glucose-6-phosphate dehydrogenase was added to half of the incubations, and these contained 4.8 ml buffer. The total assay volume was 5.0 ml. The reaction mixtures were preincubated for 90 sec in a Mickle shaking incubator at 42°C, the normal physiological temperature for birds. Metabolism was initiated by the addition of 20 μl of labeled carbaryl in ethanol, and each incubation contained 2.4 mg carbaryl. Incubations were carried out at 42°C for 5 min and the metabolism was terminated by extraction with 3 ml diethyl ether. The incubation medium was then acidified by the addition of 0.1 ml of 2 *M* HCl to facilitate the removal of any acid metabolites. Distilled water (1.0 ml) was also added to the incubation medium to aid separation of layers. This was followed by two further extractions with diethyl ether. After extraction, the combined ether extracts were dried over anhydrous sodium sulphate to minimize hydrolysis.

Extracts were blown down just to dryness with nitrogen and the residues dissolved in ethyl acetate and transferred to TLC plates (silica gel 60, 20 × 20 cm). Standards of [^{14}C]carbaryl were applied as markers. Plates were developed in a diethyl

ether:hexane mixture (4:1, v/v), and labeled carbaryl and metabolites were located using a Berthold automatic TLC linear analyzer.

RESULTS

Oral administration of 200 mg/kg carbaryl was lethal to 4 out of 12 partridges pretreated with 167 mg/k malathion. Death occurred 2 to 3 hr after dosing with carbaryl. A further 6 out of 12 birds showed symptoms of ChE poisoning. Only 2 of the 12 malathion-pretreated birds showed no visible signs of toxicity. By contrast, birds given either malathion or carbaryl alone showed no visible symptoms of toxicity. The first signs of neurotoxicity observed in birds which received both pesticides were salivation, weakness, and increased respiration. In those birds more severly affected, muscular twitching and body tremors occurred, and progressed to an inability to stand, wing-beat convulsions, cloacal discharge, and paralysis.

The serum BuChE activity which occurred following treatment with malathion alone, carbaryl alone, or malathion and carbaryl in combination is shown in Fig. 2. The time scale refers to hours after dosing with malathion in oil or with oil alone. Malathion pretreatment resulted in a highly significant ($P < 0.001$, relative to zero time activity) 53 ± 3% inhibition of serum BuChE activity 1 hr after dosing, at which time carbaryl was administered to birds which received both pesticides. The inhibition had remained at this level 3 hr later. In birds pretreated with corn oil, carbaryl administration led to a 75% inhibition of serum BuChE activity 1 hr later and an 81% depression of this activity 3 hr later (2 and 4 hr after corn oil pretreatment, respectively). Pretreatment with malathion led to an 85% inhibition 1 hr after dosing with carbaryl and a 91% inhibition 3 hr after carbaryl administration. Although malathion pretreatment produced slightly more inhibition of BuChE activity than corn oil pretreatment, the difference

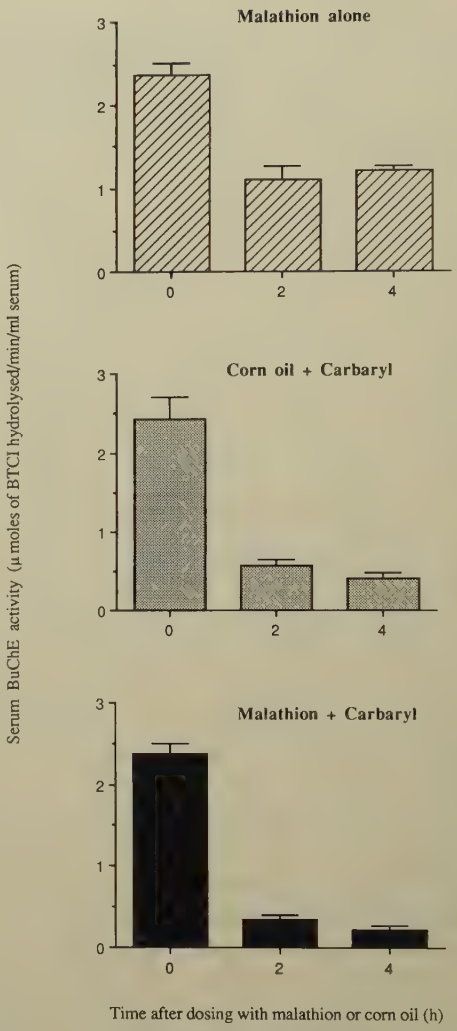

FIG. 2. *Inhibition of serum butyrylcholinesterase (BuChE) activity in partridges treated with malathion alone* (n = *8*), *corn oil and carbaryl* (n = *10*), *or malathion and carbaryl* (n = *10*).

was not significant when based on mean changes in activity.

Brain AChE activities for the different treatments are shown in Fig. 3. At 4 hr after dosing with malathion alone, a small nonsignificant 5% elevation of brain AChE activity occurred compared to control birds dosed with corn oil alone. Dosing corn oil-pretreated birds with carbaryl produced a significant ($P < 0.001$) 56% reduction in brain AChE activity compared with controls. Malathion and carbaryl given in com-

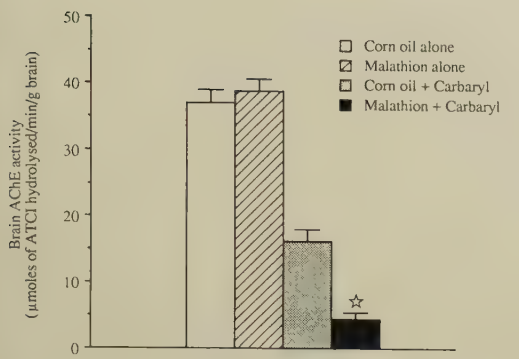

FIG. 3. *Brain acetylcholinesterase (AChE) activity in partridges following dosing with corn oil alone* (n = 9), *167 mg/kg malathion alone* (n = 8), *200 mg/kg carbaryl alone* (n = 8), *or malathion and carbaryl in combination* (n = 8). *Significance level (Student's* t *test) was derived from activity levels for malathion and carbaryl-treated birds vs birds treated with carbaryl alone:* *P < 0.001.

bination produced an 88% reduction in brain AChE, which is significantly greater ($P < 0.001$) than that produced by carbaryl alone.

The concentrations of carbaryl detected by HPLC in the serum and brain were found to be 2- and 7.5-fold greater, respectively, in birds pretreated with malathion (Table 1), although these differences were not significant.

The investigation of the mechanism responsible for the potentiation of carbaryl toxicity by malathion showed that in the presence of NADPH, two metabolites (designated as metabolites I and II) were produced from the metabolism of [^{14}C]carbaryl by microsomes from control birds. These metabolites are indicated on the examples of linear analyzer chromatograms obtained following TLC (Fig. 4). However, metabolism of carbaryl by microsomes from birds pretreated with malathion was substantially less. The relative proportions of carbaryl and metabolites located on the TLC chromatograms (expressed as μg carbaryl equivalents or as a percentage of the total ^{14}C) for the microsomal fraction from control and malathion-treated partridges are shown in Table 2.

TABLE 1

Carbaryl Concentration in Serum (n = 6) and Brain (n = 4) for Partridges Pretreated with either Corn Oil or Malathion

Treatment	Serum carbaryl concentration (μg/ml serum)	Brain carbaryl concentration (μg/g brain)
Corn oil + carbaryl	1.28 ± 0.45	0.34 ± 0.12
Malathion + carbaryl	2.51 ± 1.22	2.55 ± 1.16

In the absence of NADPH, no metabolic products were found. Carbaryl metabolism by the microsomal fractions from control birds was substantially and significantly greater than that shown by microsomal fractions from malathion-treated partridges. Control microsomes metabolized 46% of the carbaryl present in the incubation mixture, whereas microsomes from malathion-treated birds only metabolized 3% of the carbaryl (Table 2).

DISCUSSION

Pretreatment with malathion led to a considerable amplification of carbaryl toxicity to partridges. Thus, 4 out of 12 malathion-treated birds died within 3 hr of receiving carbaryl and a further 6 showed symptoms of anticholinesterase action. None of the untreated birds showed these symptoms. These effects were related to the degree of inhibition of brain cholinesterase. Birds that received carbaryl alone showed 56% inhibition of brain AChE, while those pretreated with malathion showed 88% inhibition. The effect of the combination of these two compounds on brain AChE, the principal site of action for carbaryl, was substantially more than additive, since malathion alone produced no inhibition of this enzyme. Also, there was a clear potentiation of toxicity in the sense that neither of the insecticides on its own produced any toxic symptoms, let alone death, while the combination produced clear symptoms of toxicity in 10 birds out of 12.

The malathion-pretreated birds showed

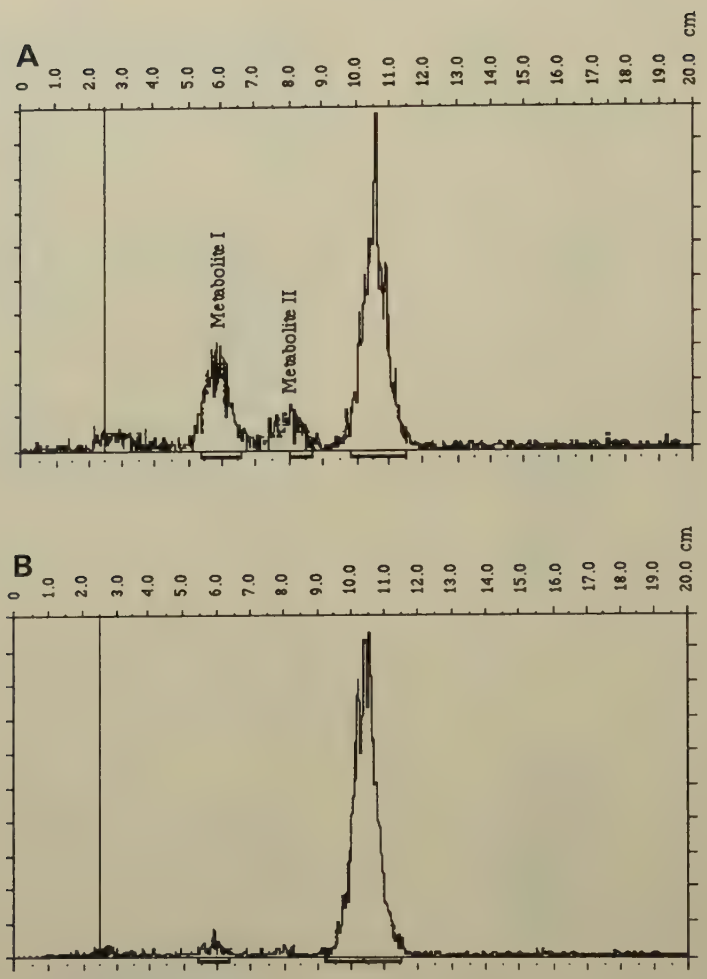

FIG. 4. *Linear analyzer chromatograms showing the metabolism of carbaryl by (A) liver microsomes from corn oil-treated partridges in the presence of NADPH, and (B) liver microsomes from carbaryl treated-partridges in the presence of NADPH.*

over 7.5-fold higher carbaryl residues in the brain than did birds that received carbaryl alone. This may provide a reasonable explanation for the difference in toxicity between these two groups of birds. The studies on metabolism by hepatic microsomes suggest that the relatively high level of carbaryl in the brains of malathion-treated birds is the consequence of a failure of metabolic detoxication. HMOs have a critical role in the detoxication of carbaryl and other carbamate insecticides in vertebrates and in insects (14). HMO inhibitors, such as piperonyl butoxide, can strongly synergize the toxicity of compounds of this type in insects (15). In comparison with control birds, the very slow NADPH-dependent oxidative metabolism of carbaryl found in birds pretreated with malathion suggests a loss of over 90% of the oxidative capacity of individuals treated with this OP. Several studies using mammalian systems *in vitro* suggest that the dominant route of carbaryl detoxication is through oxidative metabolism, with esteratic hydrolysis providing only a minor route (16, 17).

Loss of HMO activity has been related to the process of oxidative activation of OP

TABLE 2

Comparison of Major Metabolites from TLC Linear Analyzer Chromatograms for Microsomes from Control (n = 4) and Malathion-Treated (n = 5) Partridges Following Incubation with NADPH

	Control		Malathion-treated	
	μg Carbaryl equivalents	% of total ^{14}C	μg Carbaryl equivalents	% of total ^{14}C
Carbaryl	0.98 ± 0.13	54.2 ± 6.21	1.82 ± 0.07*	97.3 ± 0.98*
Metabolite I	0.68 ± 0.08	38.2 ± 5.02	0.04 ± 0.01*	2.03 ± 0.67*
Metabolite II	0.13 ± 0.03	7.65 ± 1.69	0.02 ± 0.01*	0.85 ± 0.43*

Note. Data are expressed as μg carbaryl equivalents (corrected for recovery) and as percentages. Significance level (Student's *t* test) for treated vs control; $*P < 0.001$.

compounds, such as parathion, which contain a thion group (18–20). The loss of HMO activity has been related to the release of atomic sulfur during oxidative desulfuration by cytochrome P450. In this state, sulfur is a highly reactive electrophile which becomes covalently bound to the protein moiety of cytochrome P450 (21). Thus, it seems reasonable to suggest that in this study an inhibition of cytochrome P450 has occurred during the oxidative activation of malathion to malaoxon by a similar mechanism to that described above. A loss of HMO activity following exposure may subsequently lead to a slowing in the detoxication of carbaryl, resulting in greater inhibition of brain AChE activity due to an accumulation of relatively large amounts of carbaryl in the nervous system compared to that found in birds exposed to carbaryl alone.

It is thought that hydrolysis by B-esterases (such as BuChE) in the blood represents the first line of defense against carbaryl toxicity, minimizing its availability and reducing its potential effects on nervous system AChE (22). The liver represents the second, and most important, line of defense. Any carbaryl not detoxified by the liver, coupled with the saturation of serum B-esterases, may result in free carbaryl in the circulation which is then available to inhibit AChE of the nervous system. In both control and malathion-pretreated birds, carbaryl administration resulted in a high level of saturation (carbamylation) of serum BuChE. However, malathion pretreatment had itself produced approximately 50% inhibition of serum BuChE prior to carbaryl administration. Thus, less of this enzyme was available for the sequestration and/or hydrolysis of carbaryl. It is expected, therefore, that more free carbaryl would pass through the blood to the central nervous system in the case of malathion-pretreated birds. The observed potentiation of carbaryl toxicity in the partridge following exposure to malathion is likely to be the result of two independent processes: suicide inhibition by malathion of one or more forms of cytochrome P450 (especially of the hepatic microsomal monooxygenase system) responsible for carbaryl detoxication, coupled with a decrease in availability of serum B-esterases. However, for reasons discussed earlier, it is probable that the former of these two processes is of far greater importance than the latter.

A comparison of Rf values and Rf values relative to carbaryl with those from a study by Strother (23) strongly suggests that the major metabolite (I) was 1-naphthyl-*N*-hydroxymethylcarbaryl and that metabolite II was 5-hydroxycarbaryl, although this conclusion must be confirmed using GC–MS analysis.

The studies described here were performed in the laboratory using relatively high doses of carbaryl with the aim of testing a mechanism that may lead to a potentiation of toxicity. It is unlikely that birds

would be exposed to such high levels of this carbamate insecticide under normal conditions of use in the field. The results do, however, raise more questions about the possible potentiation of toxicity of certain other insecticides which are detoxified by HMOs in situations in which birds in the field have been exposed to OP insecticides containing a thion group. In the first place, a considerable number of OPs of this type are currently used in agriculture (and sometimes also in forestry) around the world. These include dimethoate, chlorpyriphos, diazinon, pirimiphos-methyl, malathion, azinphos-methyl, fenthion, fonofos, disyston, and phorate. A number of field studies have established that the normal use of OP insecticides in agriculture or forestry can lead to substantial inhibition of brain and/or blood ChE activity in wild birds (24–26). Further, annual reports of the Wildlife Incident Investigation Scheme (WIIS) produced by the Ministry of Agriculture, Fisheries, and Food in Great Britain have detailed a number of incidents in which birds or mammals have died from OP or carbamate poisoning following the recommended use of chemicals for agricultural or veterinary purposes (8–10). OPs implicated in these incidents have included disulfoton, fonofos, triazofos, famfur, dimethoate, carbophenothion, chlorfenvinfos, demeton-*S*-methyl, and trichlorfon, the first six of which contain thion groups. From this evidence, it is clear that birds may become exposed to these compounds when they are used on agricultural or forestry land. Indeed, in a study on agricultural land, it was shown that a normal application of demeton-*S*-methyl led to inhibition of blood ChE of nestling tree sparrows (*Passer montana*) by up to 70% (24, 27).

Returning to incidents reported by WIIS, there are a number of examples of lethal poisoning of wild birds and mammals following recommended use of carbamate insecticides and molluscicides (8–10). Compounds involved include carbofuran, aldicarb, bendiocarb, and methiocarb. While all of these may have been straightforward cases of poisoning by the carbamates alone, it is also possible that certain individuals may have been particularly susceptible to the carbamates due to prior or simultaneous exposure to OPs containing thion groups. When OP insecticides of this type are used in the same field or the same area as that where highly toxic carbamates have been applied (e.g., in a granular formulation), there is a possibility that this type of interaction may occur. This possibility is currently being investigated as an extension of the present study.

Loss of HMO activity as a consequence of oxidative desulphuration may lead to increased toxicity of insecticides other than carbamates which are also detoxified by this enzyme system (28). Examples include pyrethroid insecticides and certain OPs which are not subject to activation by HMOs, e.g., chlorfenvinphos, dichlorvos, mevinphos, and trichlorphon. Where HMOs plays a major role in detoxication (this may be apparent from synergistic effects of HMO inhibitors), potentiation of toxicity following oxidative desulphuration may be expected. Cases such as this deserve further investigation.

The major difficulty in any study of interactive effects of pesticides and other pollutants lies in establishing whether they occur under field conditions. Here, there is a case for developing simple, specific, sensitive, and nondestructive biomarker assays which are user-friendly (29, 30). By the use of such techniques in well-designed field or semi-field studies, it should be possible to identify situations in which exposure to two chemicals leads to a greater than additive response as the consequence of a toxicokinetic interaction such as that described here.

ACKNOWLEDGMENTS

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Characterization, Purification, and Reconstitution of an Inducible Cytochrome P450-Dependent Triasulfuron Hydroxylase from Wheat[1,2]

FREDERIC W. THALACKER,[3] HARLEY R. SWANSON, AND D. STUART FREAR

United States Department of Agriculture, Agricultural Research Service, Biosciences Research Laboratory, State University Station, Fargo, North Dakota 58105-5674

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This report describes the further purification and characterization as well as the solubilization and reconstitution of an inducible cytochrome P450 monooxygenase responsible for triasulfuron hydroxylation in wheat. Improved procedures for increasing the levels of expressed microsomal cytochrome P450 and triasulfuron hydroxylase activity in etiolated wheat seedling shoot tissues are described. Cytochrome P450 concentrations and triasulfuron hydroxylase activities were increased to 1.17 nmol mg^{-1} protein and 2.87 nmol min^{-1} mg^{-1} protein, respectively, with an improved enzyme assay system and the use of sucrose density gradient-purified microsomal preparations from naphthalic anhydride- and ethanol-induced coleoptile tissues. A pH optimum between 7.0 and 7.5 in potassium phosphate buffer and an apparent K_m of 24 μM for triasulfuron were determined. Dependence of triasulfuron hydroxylation on cytochrome P450 was established with cytochrome P450 inhibitors (tetcyclacis, paclobutrazol, and piperonyl butoxide), reduced CO difference spectra, light-reversible CO inhibition, type I substrate and type II inhibitor binding spectra, and by inhibition with polyclonal antibodies raised against a partially purified fraction of wheat NADPH cytochrome c reductase (EC 1.6.2.4). Microsomal NADPH cytochrome c reductase and cytochrome P450 were solubilized with detergent (1% Chaps, v/v), separated by affinity chromatography on 2′,5′ADP-Sepharose 4B, and purified by DEAE ion exchange chromatography. Triasulfuron hydroxylase activity was reconstituted by chromatography of the purified NADPH cytochrome c reductase and cytochrome P450 fractions on hydroxyapatite. In preliminary studies with the reconstituted enzyme system, triasulfuron hydroxylase activity was 6% of the induced microsomal activity. © 1994 Academic Press, Inc.

INTRODUCTION

In addition to their role in the oxidative metabolism of a variety of different endogenous substrates (1), plant cytochrome P450 monooxygenases are also responsible for many of the oxidative reactions used by plants to detoxify herbicides and other xenobiotics (2–5).

Several examples of reconstituted cytochrome P450-dependent enzymes from plants that metabolize endogenous substrates have been reported (6–11). However, while there is strong evidence that plant cytochrome P450-dependent enzymes are responsible for the selective action and metabolic detoxification of herbicides used for selective postemergence weed control in wheat (12–15), there appear to have been no reports of reconstituted cytochrome P450-dependent enzymes responsible for the metabolism of these herbicides.

There are several possible reasons for the limited progress that has been made in the

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[2] Presented in part at the 204th American Chemical Society National Meeting, Washington, DC, August, 1992.

[3] Current address: Hazleton Laboratories, Agricultural Chemistry, Plant and Animal Metabolism, Madison, WI.

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isolation and characterization of specific cytochrome P450s responsible for herbicide metabolism in wheat and other plants. Two important reasons are low concentrations and activities of constitutive cytochrome P450-dependent enzymes and the absence of active reconstituted microsomal enzyme systems necessary to determine the activity and specificity of isolated cytochrome P450 isoforms.

A number of sulfonylurea herbicides, including chlorsulfuron[4] and triasulfuron, have been developed for the postemergence control of broadleaf weeds and the suppression of some grassy weeds in cereal crops (16, 17). In wheat and corn leaf tissues, chlorsulfuron is metabolized and detoxified *in vivo* by hydroxylation at the 5-position on the chlorophenyl ring (18, 19). Recent studies with naphthalic anhydride- and ethanol-induced etiolated wheat seedling microsomal preparations have also shown that chlorsulfuron and triasulfuron are both hydroxylated *in vitro* at the 5-position on their respective substituted phenyl rings (12).

In order to further characterize the inducible cytochrome P450-dependent enzyme(s) responsible for triasulfuron hydroxylation and to help facilitate the identification of the gene(s) that regulate triasulfuron hydroxylase activity and tolerance in wheat, the objectives of the present study were to increase the levels of triasulfuron hydroxylase and cytochrome P450 in etiolated wheat seedling microsomal preparations, to optimize conditions for the induction and assay of triasulfuron hydroxylase activity, and to develop a reconstituted cytochrome P450 system with triasulfuron hydroxylase activity.

Further evidence is presented that an inducible cytochrome P450-dependent hydroxylase is responsible for triasulfuron oxidation and detoxification in wheat. Improved conditions for the induction of etiolated wheat seedling cytochrome P450s, for increased triasulfuron microsomal hydroxylase activity, and the partial purification and reconstitution of triasulfuron hydroxylase activity from wheat microsomes are reported. To our knowledge, this report together with a recent report of 2,4-D oxidation by a reconstituted cytochrome P450 system from tulip (20) represent the first reports of herbicide metabolism by reconstituted plant cytochrome P450 systems.

MATERIALS

3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Chaps), dithiothreitol (DTT), and sucrose were from Research Organics (Cleveland, OH). Sephadex G-25M PD-10 columns, 2′,5′ADP-Sepharose 4B, and the fast protein liquid chromatography (FPLC) system were from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ). Protein PAK DEAE packing (40 μmol) was from Waters (Milford, MA). Econo-Pac hydroxyapatite cartridges were from Bio-Rad (Richmond, CA). Reduced Triton X-100 and 1,8-naphthalic anhydride (NA) were from Aldrich (Milwaukee, WI). Immunopure (A) IgG purification kits and protein determination kits were from Pierce Chemical Co. (Rockford, IL). Thin layer chromatography (TLC) plates were from Whatman Lab Sales (Hillsboro, OR). Ultrafiltration membranes (Diaflo YM10) were from Amicon (Beverly, MA). The CO (99.3%) was from Scott Specialty Gases, Inc. (Plumsteadville, PA). All other chemicals were from Sigma Chemical Co. (St. Louis, MO). [*phenyl*-U-^{14}C]triasulfuron

[4] Abbreviations used: Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; EtSH, 2-mercaptoethanol; FPLC, fast protein liquid chromatography; DMSO, dimethyl sulfoxide; BSA, bovine serum albumin; NA, 1,8-naphthalic acid anhydride; TLC, thin-layer chromatography; triasulfuron, 1-(2-chloroethoxyphenylsulfonyl)-3-(6-methoxy-4-methyl-1,3,5-triazin-2-yl)urea; chlorsulfuron, 1-(2-chlorophenylsulfonyl)-3-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)urea; 5-OH triasulfuron, 1-(2-chloroethoxy-5-hydroxyphenylsulfonyl)-3-(6-methoxy-4-methyl-1,3,5-triazin-2-yl)urea; RTX-100, reduced Triton X-100; EDTA, ethylenediamine tetraacetic acid, disodium salt; HTP, hydroxyapatite chromatography cartridge; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

(49.5 μCi/mg, 19.9 μCi/μmol) was provided by the Ciba-Geigy Corp. (Greensboro, NC). Radiolabeled substrates were purified to greater than 99% radiochemical and chemical purity by TLC. Triasulfuron and [5-hydroxyphenyl]triasulfuron reference compounds were provided by Ciba-Geigy Corp.

METHODS

Plant materials. Wheat (*Triticum aestivum* L., var. Olaf) seeds were sown in vermiculite, subirrigated with 0.5 m*M* $CaSO_4$, and germinated in the dark at 21°C. After 66–114 hr, etiolated shoots (0.75–1.5 cm) were excised and used directly or separated into coleoptile and leaf tissues for the preparation of microsomes. All excised tissues were at the same or similar stage of growth based on the size of the shoot, except where specifically noted.

Induction treatments. Microsomal triasulfuron hydroxylase activity and cytochrome P450 were induced as described previously (12). NA was applied directly as a seed dressing (0.25%, w/w). Ethanol was applied by subirrigation as a 0.5–15% (v/v) solution in 0.5 m*M* $CaSO_4$ 24 hr before the etiolated shoots were excised.

Microsome isolation. Microsomes were isolated by differential centrifugation from 6 to 80 g of etiolated shoots, leaves, or coleoptiles and stored as previously reported (12). A cocktail of protease inhibitors (21) was sometimes added to both the tissue homogenization and microsomal suspension buffers to reduce the possible loss of enzyme activity. However, further studies established that the addition of protease inhibitors did not affect enzyme activity.

Sucrose density gradient microsome purification. Microsomal fractions obtained by differential centrifugation were purified further by sucrose density gradient centrifugation. After gel filtration on a Sephadex G-25 PD-10 column to remove glycerol, 2.5-ml aliquots of suspended microsomes (2–5 mg protein/ml) in 0.1 *M* potassium phosphate buffer, pH 7.5, with 2.5 m*M* DTT were layered carefully on top of a discontinuous sucrose gradient (from the bottom) of 3.5 ml 45% sucrose, 3.5 ml of 35% sucrose, and 2.5 ml of 10% sucrose in the suspension buffer. After centrifugation in a SW41 rotor (Beckman) for 2 hr at 35,000 rpm (100,000*g*), the microsomal membrane layer at the 10/35% sucrose interface (1–1.5 ml) was carefully removed with a pipette and placed on a Sephadex G-25 PD-10 column equilibrated with suspension buffer. Eluted Sephadex G-25 microsomal fractions in suspension buffer were than adjusted to 10% (v/v) glycerol, frozen with dry ice, and stored at −70°C.

Triasulfuron hydroxylase assays. Standard triasulfuron hydroxylase assays contained 40 nmol of [^{14}C]triasulfuron (ca. 2×10^5 dpm) with a sp act of 2.25 μCi/μmol together with 0.1–0.6 mg of microsomal protein, 0.1 *M* potassium phosphate buffer (pH 7.5), 2.5 m*M* DTT, 1 m*M* NADPH, and 1% (w/v) globulin-free bovine serum albumin (BSA) in a final volume of 1.0 ml. Reactions were initiated by the addition of substrate and NADPH and were incubated at 25°C for 10–15 min. Control reactions were run without NADPH. Under these assay conditions, enzyme activity exhibited steady-state kinetics, was proportional to microsomal protein concentration, and linear with respect to time. The pH optimum for enzyme activity was 7.0–7.5 in 0.1 *M* potassium phosphate buffer and the apparent K_m for triasulfuron was 24 ± 3 μ*M* (data not shown).

Reactions were terminated by adjusting the pH to 5.0 with 50 μl of 4.4 *N* H_3PO_4. After the addition of 3 ml of H_2O, the acidified reaction mixtures were extracted three times with 3.0 ml ethyl acetate and the combined extracts were taken to dryness under vacuum at 43°C. The concentrated ethyl acetate extracts were dissolved in 0.2 ml of 90% aqueous MeOH and analyzed by TLC on Whatman LK6F plates. The TLC solvent system used for the isolation and analysis of the microsomal oxidation product 1-(2-chloroethoxy-5-hydroxyphenylsulfonyl)-3-(6-methoxy-4-methyl-1,3,5-triazin-2-yl)urea (5-OH triasulfuron) was benzene:

acetone:formic acid (30:10:1, v/v). Separated 5-OH triasulfuron and parent triasulfuron were located by visualizing cochromatographed standards under ultraviolet light or by scanning on an image-analyzing radioactivity detector (Bioscan System 200 Imaging Scanner, Bioscan, Inc., Washington, DC). Oxidation product zones were scraped off the TLC plates and quantitated by liquid scintillation spectrometry with Universol cocktail (ICN Biomedicals, Inc.).

Spectrophotometric assays. Reduced cytochrome P450 carbon monoxide difference spectra, NADPH cytochrome c reductase activity, and protein concentrations were determined as reported previously (12).

Substrate and inhibitor binding spectra. Cytochrome P450 binding spectra were determined with coleoptile microsomal preparations under oxidized conditions as described by Jefcoate (22). From 0.8 to 1.5 mg of microsomal protein (386–699 pmol cytochrome P450) was suspended in 0.1 *M* potassium buffer (pH 7.5) with or without 1 m*M* DTT. Triasulfuron or tetcyclacis dissolved in a small volume (10 μl) of dimethyl sulfoxide (DMSO) were added to the sample cuvette and DMSO alone was added to the reference cuvette. DMSO levels were 1.0% (v/v) in a total volume of 1.0 ml. Spectra were obtained with a Model 219 Cary spectrophotometer (0.01 to 0.05 A full scale) at room temperature.

Inhibition studies. Several tests are necessary to establish whether microsomal oxidation reactions are dependent on cytochrome P450. A requirement for oxygen and NADPH in the hydroxylation of triasulfuron by wheat microsomal preparations has been reported (12). In the present studies, light-reversible CO-inhibition of triasulfuron hydroxylation was also demonstrated by injecting 3 ml of CO into a sealed 8.5-ml vial (40% CO, v/v) containing air and 1 ml of the microsomal reaction mixture with NADPH (1 mg/1.5 mg protein). After equilibration for 30 min at 4°C in the dark, additional NADPH (1 μmol) and [^{14}C]triasulfuron (40 nmol) were added to initiate the reaction. Samples were incubated ± exposure to 3000 μE m^{-2} sec^{-1} of light for 15 min at 25°C. A slide projector lamp (300 W) on full power was placed 1 or 2 inches from the samples in a water bath and used as the light source. Control samples were injected with 3 ml of N_2 in place of CO and also exposed to light.

Studies with selected inhibitors of cytochrome P450 monooxygenases, including cytochrome c, piperonyl butoxide, tetcyclacis, and paclobutrazol, were conducted as reported (12). Microsomal reaction mixtures were equilibrated for 10 min at 4°C in the presence of the inhibitor. The reaction was initiated by the addition of NADPH and [^{14}C]triasulfuron and incubated for 15 min at 25°C. Inhibitors were dissolved in buffer or added in DMSO to provide a final concentration of ≤1% (v/v) DMSO. This concentration of DMSO did not affect enzyme activity.

Antibody formation, isolation, and activity. NADPH cytochrome c reductase polyclonal antibodies (rabbit) were raised against a partially purified NADPH cytochrome c reductase fraction from wheat microsomes.

Resuspended microsomes (1.7 g protein) were solubilized in buffer A [0.1 *M* potassium phosphate buffer, pH 7.5, 2.5 m*M* DTT, 10% (v/v) glycerol, and 1% (w/v) Chaps]. Solubilized microsomal protein (1.1 g) was applied to a preparative DEAE Protein-Pak (40 μm Waters) FPLC column (2.5 × 10 cm) in buffer A and eluted at a flow rate of 2 ml/min with a stepped linear gradient of KCl in buffer B (1.0 *M* KCl in buffer A). The gradient program was 0% B for 20 min, 0–40% B (0.0–0.4 *M* KCl) in 25 min, 40–100% B (0.4–1.0 *M* KCl) in 5 min, and 100% B (1.0 *M* KCl) for 3 min. A broad peak of NADPH cytochrome c reductase activity (0.25–0.75 *M* KCl) was collected, concentrated by pressure ultrafiltration in a stirred cell, and applied to a 2′,5′ ADP-Sepharose 4B column (2.5 × 5 cm) in buffer A. Adsorbed NADPH cytochrome c reduc-

tase was eluted by 1.1 m*M* $NADP^+$ in buffer A. The eluted enzyme was concentrated by pressure ultrafiltration in a stirred cell and rechromatographed on the DEAE Protein-Pak (40 μm) FPLC column (2.5 × 10 cm) with the same stepped linear KCl gradient noted above. A sharp narrow peak of NADPH cytochrome c reductase activity (0.3–0.4 *M* KCl) was collected, concentrated by ultrafiltration, and purified further by chromatography on a (10 × 300-mm) Superose 12 FPLC gel filtration column (Pharmacia) with buffer C [0.05 *M* potassium phosphate buffer (pH 7.5), 10% (v/v) glycerol, 0.2% (v/v) RTX-100, 0.1 m*M* EDTA, and 1.5 m*M* 2-mercaptoethanol (EtSH)] at a flow rate of 0.5 ml/min. A broad peak of NADPH cytochrome c reductase activity was separated into early, middle, and late eluting fractions and analyzed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). SDS–PAGE analysis of the middle fraction showed that three closely sized proteins (M_r 78,200, 81,100, and 84,900, $n = 8$) were present. Three isozymes of NADPH cytochrome c reductase (M_r 80,000, 82,000, and 84,000) have been reported recently in Jerusalem artichoke (23). Since the Superose 12 middle fraction contained the majority of NADPH cytochrome c reductase activity, this partially purified fraction (in buffer C) was exchanged for phosphate-buffered saline (137 m*M* NaCl, 2.7 m*M* KCl, 4.3 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4) with 0.2% (v/v) RTX-100, 10% (v/v) glycerol, and 0.1 m*M* EDTA and used to produce polyclonal antibodies. After collection of preimmune serum, rabbit-raised polyclonal antibodies were produced by subcutaneous injection of the partially purified NADPH cytochrome c reductase (ca. 450 μg protein) with Freund's complete adjuvent followed by three subsequent injections (ca. 450 μg protein each) at 2-week intervals in Freund's incomplete adjuvent (24). Two weeks after the final injection, the rabbit was sacrificed and blood was collected by heart puncture. Serum was separated from clotted material after storage for several hours at room temperature, divided into smaller volumes, and stored at −70°C.

The antibody fractions from both preimmune and immunized serums were purified by affinity chromatography with a Pierce Immunopure (A) IgG purification kit according to the manufacturers instructions except that the diluted serum (1:1 with binding buffer) was recirculated through the protein A column several times. Following collection of the IgG antibody fraction, anti-NADPH cytochrome c reductase activity was assayed. After incubating the purified IgG NADPH cytochrome c reductase antibody fraction with coleoptile microsomes for 1 hr at 4°C, NADPH cytochrome c reductase and triasulfuron hydroxylase activity were measured as described under Methods.

Solubilization and purification of NADPH cytochrome c reductase and cytochrome P450 for reconstitution. Resuspended microsomes (5–15 mg protein/ml) in 0.1 *M* potassium phosphate buffer (pH 7.5), 2.5 m*M* DTT, and 10% (v/v) glycerol were solubilized with 1% (w/v) Chaps for 1 hr at 4°C with gentle stirring. Residual insoluble material was removed by ultracentrifugation (2 hr at 100,000*g*). Solubilized proteins were recirculated through a 2′,5′ ADP-sepharose 4B affinity column (2.5 × 5 cm) at a flow rate of 1.5 ml/min for 1 or 2 hr at 4°C. The 2′,5′ ADP-Sepharose 4B column was washed with 30–40 ml of buffer A, then washed with 20–30 ml of buffer D [0.2 *M* potassium phosphate (pH 7.5), 10% (v/v) glycerol, 0.1 m*M* EDTA, 0.2% (v/v) RTX-100, 1.5 m*M* EtSH], and washed again with 15–20 ml of buffer A. Adsorbed NADPH cytochrome c reductase activity was eluted with 10–15 ml of 1.1 m*M* $NADP^+$ in buffer A, concentrated by pressure ultrafiltration in a stirred cell, and stored at −70°C. Enzyme activity was stable for several weeks when stored at −70°C.

The nonbinding 2′,5′ ADP-sepharose 4B eluate fraction (putative cytochrome P450) was exchanged for buffer A by gel filtration

on Sephadex G-25 PD-10 columns. Aliquots (ca. 7 ml each) of the Sephadex G-25 PD-10 column eluate were chromatographed on a DEAE Protein- Pak (40 μm) FPLC column (2.5 × 10 cm) equilibrated with buffer A and eluted at a flow rate of 2.0 ml/min with the same stepped linear KCl gradient program as that described for NADPH cytochrome c reductase purification. The major 405-nm absorbing putative cytochrome P450 fraction that eluted between 0.1–0.16 *M* KCl was concentrated by ultrafiltration and stored at −70°C.

Reconstitution of triasulfuron hydroxylase activity. Triasulfuron hydroxylase activity was reconstituted following detergent solubilization and partial purification of microsomal proteins from NA/EtOH-induced wheat shoots or coleoptiles. Isolated soluble cytochrome P450 and NADPH cytochrome c reductase fractions described above were desalted individually on Sephadex G-25 PD-10 columns with 0.01 *M* potassium phosphate buffer (pH 7.5), 10% (v/v) glycerol, 0.2% (v/v) RTX-100, and 2.5 m*M* DTT and then chromatographed separately by FPLC on two Econo-Pac hydroxyapatite chromatography (HTP) cartridges (connected in series) with 0.01 *M* potassium phosphate (pH 7.5), 10% (v/v) glycerol, and 2.5 m*M* DTT as the buffer. Adsorbed cytochrome P450 and NADPH cytochrome c reductase fractions were each eluted with 0.5 *M* potassium phosphate buffer (pH 7.5), 10% (v/v) glycerol, and 2.5 m*M* DTT. Each of the eluted cytochrome P450 and NADPH cytochrome c reductase fractions was concentrated by pressure ultrafiltration in a stirred cell and then with a Centricon 10 microconcentrator (Amicon) followed by reconstitution with phospholipid (System I, Table 9). An additional sample, containing both of the isolated and desalted cytochrome P450 and NADPH-cytochrome c reductase fractions, was chromatographed together on the same Econo-Pac HTP hydroxyapatite columns in series as described above and also reconstituted with phospholipid (System II, Table 9). Phospholipid concentrations (10 μg dilauroylphosphatidycholine) in the reconstituted triasulfuron hydroxylase reactions were the same as reported for a reconstituted 3,9-dihydroxypterocarpan 6a-hydroxylase from soybean (9) and a reconstituted geranoil-10-hydroxylase from *Catharanthus roseus* (25). Triasulfuron hydroxylase assays with the reconstituted enzymes were performed as described except that the final reaction volume was reduced to 200–400 μl, [^{14}C]triasulfuron-specific activity was increased, substrate concentration was reduced to 4 nmol, the reaction time was increased, and BSA was not added to the reaction mixture. Reconstituted enzymes were equilibrated for 15 min at 4°C and then incubated for an additional 10 min at 25°C with [^{14}C]triasulfuron (1.77×10^5 dpm; 19.9 μCi/μmol) before the reactions were initiated by the addition of NADPH. Reactions were stopped after 30 min and analyzed as described under Methods. Changes in the reaction conditions were designed to increase the detectable levels of [^{14}C]5-OH triasulfuron produced.

RESULTS AND DISCUSSION

Improved enzyme assay system. Previous studies showed that several changes in the microsomal triasulfuron hydroxylase assay system resulted in increased rates of hydroxylation (12). These changes included the addition of BSA, the removal of small-molecular-weight inhibitors by gel filtration (Sephadex G25), and the elimination of glycerol from the enzyme assays. In the present study, an additional increase in microsomal triasulfuron hydroxylase activity was obtained by the replacement of EtSH with DTT in the assay system. In contrast to a specific requirement for millimolar concentrations of EtSH in the hydroxylation of *trans* cinnamic acid by pea seedling microsomes (26), removal of EtSH or replacement of EtSH with DTT resulted in almost a 100% increase in triasulfuron hy-

TABLE 1
The Effect of Dithiothreitol and 2-Mercaptoethanol on Microsomal Triasulfuron Hydroxylase Activity[a]

Treatment	Concn (m*M*)	Triasulfuron hydroxylase (pmol min^{-1} mg^{-1} protein)	% Maximum activity
No addition	—	920	98
+DTT	2.5	937	100
+EtSH	2.5	522	56
+DTT + EtSH	2.5 + 2.5	500	53

[a] Microsomes from NA/EtOH-induced etiolated wheat seedling shoots.

droxylase activity (Table 1). Inhibition of enzyme activity by EtSH was unaffected by the addition of DTT to the reaction mixture.

Improved seedling growth conditions. Several factors were found that increased microsomal cytochrome P450 levels and triasulfuron hydroxylase activity. The first factor of importance was the growth of healthy wheat seedling tissues. A number of studies showed that different vermiculite lots varied greatly in their ability to support germination and seedling growth. In some lots, an inhibitory activity associated with the vermiculite caused stunting of the roots and slowing of seedling growth and development. While the specific effects of different sources of vermiculite on cytochrome P450 levels were not determined, the stunting of plant growth and development often resulted in uneven stands of germinated seedlings and variability in microsomal triasulfuron hydroxylase activity. Each new lot of vermiculite was tested for possible seedling injury.

Another factor that improved seedling growth was the volume of 0.5 m*M* $CaSO_4$ used for germination. Studies showed that 1200 ml of 0.5 m*M* $CaSO_4$ fully saturated two trays (15.5 × 11 × 6 cm) of medium-grade vermiculite that was seeded 1 cm below the surface with 32 g of wheat. Greater volumes of 0.5 m*M* $CaSO_4$ resulted in conditions that slowed seedling growth, lowered P450 levels, and resulted in lower overall experimental consistency. Saturation of the vermiculite germination media with 1200 ml of 0.5 m*M* $CaSO_4$ also provided a better estimate of EtOH concentrations when EtOH was used as an inducer.

Effect of wheat seedling age on microsomal cytochrome P450, NADPH cytochrome c reductase, and triasulfuron hydroxylase activity. Tissue age also affected cytochrome P450 levels and triasulfuron hydroxylase activity. As the age and length of seedling tissues increased, cytochrome P450 levels together with triasulfuron hydroxylase and NADPH cytochrome c reductase activities were decreased (Table 2).

TABLE 2
Effect of Wheat Seedling Age on Microsomal Cytochrome P450, NADPH Cytochrome c Reductase, and Triasulfuron Hydroxylase Activities[a]

Seedling age (hr)	Shoot length (cm)	Cyt. P450 (pmol mg^{-1} protein)[b]	NADPH cytochrome c reductase (nmol min^{-1} mg^{-1} protein)[b]	Triasulfuron hydroxylase (pmol min^{-1} mg^{-1} protein)[b]
Control[c] (3)[d]	1.0–1.8	440 ± 38 [100][e]	120 ± 8 [100]	1052 ± 157 [100]
+6 (3)	1.5–2.5	398 ± 82 [90]	105 ± 9 [88]	818 ± 125 [78]
+12 (3)	2.0–3.0	346 ± 68 [79]	105 ± 13 [88]	772 ± 160 [73]
+18 (1)	3.0–4.0	293 [67]	95 [79]	527 [50]
+24 (2)	4.0–5.0	284 ± 40 [65]	87 ± 13 [73]	416 ± 38 [40]

[a] Na/EtOH-induced microsomes from etiolated wheat seedling shoots.
[b] Means ± SD of all replications.
[c] Control seedlings germinated in the dark at 21°C for 90 hr.
[d] Number of separate experiments in parentheses.
[e] Percentage of control in brackets.

A similar relationship between increased seedling age and decreased microsomal cytochrome P450-dependent *N*-demethylase activity in etiolated cotton hypocotyl tissues has been reported (27). Because of differing rates of seedling development in the presence or absence of inducers, shoot tissues at the same or similar growth stage (based on shoot length) were selected for all microsomal studies. This is an important consideration in comparative studies and has been reported previously (12, 14). Compared with seedlings grown in the dark for 90 hr, an additional 24 hr of growth in the dark resulted in a 35% decrease in cytochrome P450 levels and a 60% decrease in triasulfuron hydroxylase activity (Table 2). NADPH cytochrome c reductase activity levels were also reduced about 30%. Decreases in cytochrome P450, NADPH cytochrome c reductase, and triasulfuron hydroxylase activities with increased tissue age may be explained partially by changing proportions of coleoptile and leaf tissue as the seedling ages. At 1–1.8 cm, NA/EtOH-treated coleoptile tissues make up about 66% of the shoot mass, but contain only about 50% of the total protein present in the shoots.

Induction of wheat microsomal cytochrome P450 levels and triasulfuron hydroxylase activity. Triasulfuron hydroxylase activity was increased up to 25-fold in wheat seedling tissues treated with a combination of NA and EtOH as inducers (Table 3). NA or EtOH induced triasulfuron hydroxylase activity 6- to 23-fold, respectively. Although NA did not appear to affect microsomal cytochrome P450 levels to any significant extent, EtOH and the combination of both NA and EtOH increased cytochrome P450 levels 33 and 50%, respectively, when compared to noninduced controls (Table 3). NADPH cytochrome c reductase activity changed only to a limited extent (<15%) with NA and/or EtOH induction. Marked differences between the approximately 1.5-fold increase in NA/EtOH-induced cytochrome P450 levels and the >25-fold increase triasulfuron hydroxylase activity suggest that a particular cytochrome P450 isoform responsible for triasulfuron hydroxylation may be highly inducible yet represents a relatively small proportion of the total cytochrome P450 pool.

Effect of EtOH concentration on triasulfuron hydroxylase activity. The effects of NA seed treatment in combination with different concentrations of EtOH on the induction of triasulfuron hydroxylase activity are shown in Table 4. Although a combination of 10% (v/v) EtOH and NA increased triasulfuron hydroxylase activity the most, NA treatments with 2% (v/v) or 5% (v/v) EtOH increased enzyme activity to almost the same extent. Concentrations of EtOH as low as 0.5% (v/v) together with NA also

TABLE 3
Induction of Wheat Microsomal Triasulfuron Hydroxylase Activity

Inducer	Cyt. P450 (pmol mg^{-1} protein)[a]	NADPH Cyt.c Red. (nmol min^{-1} mg^{-1} protein)[a]	Triasulfuron hydroxylase (pmol min^{-1} mg^{-1} protein)[a]
None (3)[b]	302 ± 7	130 ± 12	41 ± 3
NA (6)	271 ± 40	111 ± 7	253 ± 56 [6.2]
EtOH (3)	403 ± 30 [1.3][c]	120 ± 4	968 ± 57 [23.6]
NA + EtOH (14)	452 ± 56 [1.5]	118 ± 12	1038 ± 217 [25.3]

[a] Means ± SD for experiments with microsome preparations from etiolated shoots.
[b] Number of separate experiments in parentheses.
[c] Fold increase in brackets.

TABLE 4
Effect of EtOH Concentration on Induced Triasulfuron Hydroxylase Activity

Induction treatment[a]	Triasulfuron hydroxylase (pmol min^{-1} mg^{-1} protein)[b]	Fold induction
Noninduced (3)[c]	41 ± 3	—
NA (6)	253 ± 56	6.2
NA + 0.5% EtOH (2)	351 ± 74	8.6
NA + 1.0% EtOH (2)	549 ± 73	13.4
NA + 2.0% EtOH (4)	975 ± 53	23.8
NA + 5.0% EtOH (4)	989 ± 107	24.1
NA + 10.0% EtOH (14)	1038 ± 217	25.3
NA + 15.0% EtOH (2)	888 ± 17	21.7

[a] Microsomes from etiolated wheat seedling shoots. Concentrations of EtOH presented as % (v/v).
[b] Means ± SD of all replications.
[c] Number of separate experiments in parentheses.

induced triasulfuron hydroxylase activity. The combination of 15% (v/v) EtOH and NA decreased enzyme induction, possibly because of EtOH toxicity at this higher concentration.

Dependence of cytochrome P450 levels and the triasulfuron hydroxylase activity on tissue source and isolation protocol. Cytochrome P450 levels and triasulfuron hydroxylase and NADPH cytochrome c reductase activity were highest in coleoptile tissues when compared to those in leaf tissues and intact shoots (Table 5). In comparison to leaf tissues, coleoptile tissues from Na/EtOH-induced seedlings have more than twice the levels of cytochrome P450 and NADPH cytochrome c reductase activity per milligram protein and almost three times the level of triasulfuron hydroxylase activity. Species- and tissue-specific differences in microsomal cytochrome P450-dependent enzymes responsible for herbicide oxidation reactions in plants have been reported (27, 28). Levels of cytochrome P450 and NADPH cytochrome c reductase and triasulfuron hydroxylase activity were increased 23, 26, and 41%, respectively, in coleoptile tissues compared to those in intact shoot tissues. However, coleoptile tissue isolation was a time consuming and tedious operation and etiolated shoot tissues were used for most microsomal preparations.

Reasons for tissue-specific differences in cytochrome P450 levels and triasulfuron hydroxylase activity in wheat have not been determined. Lower levels of cytochrome P450 and triasulfuron hydroxylase activity in leaf tissues may be associated with the presence of interfering pigments and endogenous inhibitors (1, 12, 28, 29). It is also possible that there are differ-

TABLE 5
Dependence of Microsomal Cytochrome P450, NADPH Cytochrome c Reductase, and Triasulfuron Hydroxylase Activities on Tissue Source and Isolation Procedure

Tissue source[a] and isolation procedure	Cyt. P450 (pmol mg^{-1} protein)[b]	NADPH cytochrome c reductase (nmol min^{-1} mg^{-1} protein)[b]	Triasulfuron hydroxylase (pmol min^{-1} mg^{-1} protein)[b]
Shoot (14)[c]	452 ± 56	118 ± 12	1038 ± 217
Coleoptile (4)	555 ± 73	149 ± 6	1465 ± 77
Leaf (3)	255 ± 74	67 ± 4	513 ± 73
Shoot (density gradient) (2)	1051 ± 33	204 ± 22	2203 ± 139
Coleoptile (density gradient) (5)	1169 ± 132	246 ± 24	2872 ± 242
Leaf (density gradient) (2)	534 ± 110	142	1136 ± 137

[a] Etiolated NA/EtOH-induced wheat seedlings.
[b] Means ± SD of all replications.
[c] Number of separate experiments in parentheses.

ences in the inducibility or expression of the cytochrome P450 in coleoptile or leaf tissues, differences in processing enzymes or inhibitors, or differences in cytochrome P450 isoforms present in the two tissues.

Microsomal cytochrome P450 concentrations and triasulfuron hydroxylase activities of shoot, coleoptile, and leaf tissues were doubled after further purification by sucrose density gradient centrifugation (Table 5). Concentrated lighter microsomal membrane fractions at the 10/35% sucrose interface were easily visible and readily removed with a minimum of contamination. Similar density gradient purification studies with microsomal preparations from etiolated cotton seedling hypocotyls have been reported (27). These studies showed that cytochrome P450-dependent *N*-demethylase activity was associated primarily with a lighter "smooth" endoplasmic reticulum fraction. Density gradient-purified wheat seedling microsomal fractions generally contained approximately 75% of the cytochrome P450 but only 25% of the total protein applied to the gradient. After density gradient purification, cytochrome P450 levels in NA/EtOH-induced microsomal preparations from either intact shoots or coleoptiles were four- to fivefold greater than those previously reported in wheat (12). Even though cytochrome P450 levels in plants are generally much lower than those reported in animals (1, 13), the levels shown in Table 5 for density gradient-purified wheat coleoptile microsomes (1.2 nmol mg^{-1} protein) are approaching the same order of magnitude reported for cytochrome P450 in phenobarbitol-induced rat liver microsomes (30).

Alterations in seedling growth conditions, the induction of enzyme activity, and microsomal purification, together with improvements in the enzyme assay system, have resulted in almost a ninefold increase in triasulfuron hydroxylase activity and over a fourfold increase in cytochrome P450 levels compared with those of induced microsome preparations from etiolated wheat seedling shoot tissues reported previously (12).

Inhibition studies. Light reversibility of CO inhibition is an established criteria for determining whether or not an enzymatic reaction is dependent on cytochrome P450. Triasulfuron hydroxylase activity was inhibited almost 50% by CO in the dark and exposure to light restored activity to 92% of the control (Table 6).

In previous studies (12), it was demonstrated that triasulfuron hydroxylase activity was dependent upon NADPH and O_2. These studies also showed that triasulfuron hydroxylase activity was inhibited by piperonyl butoxide and the plant growth regulators tetcyclacis and paclobutrazol. Cytochrome c, an effective electron acceptor for NADPH cytochrome P450 reductase, was also a potent inhibitor of triasulfuron hydroxylase activity (12). The results of similar inhibition studies with isolated coleoptile microsome preparations are shown in Table 7. Microsome preparations isolated from coleoptile tissues were more sensitive to inhibition than microsomes prepared previously from shoots that contained both leaf and coleoptile tissues (12).

Inhibitor binding studies with tetcyclacis showed strong type II spectra and provided additional evidence that triasulfuron hydroxylase activity was dependent on cytochrome P450 (Fig. 1A). Weak type I tria-

TABLE 6
Light-Reversible CO Inhibition of Triasulfuron Hydroxylation by Etiolated Wheat Seedling Microsomes[a]

	Triasulfuron hydroxylase activity (pmol min^{-1} mg^{-1} protein)[b]	% Control
Control	1383 ± 146	100
CO Dark	704 ± 91	51
CO Light	1272 ± 193	92

[a] NA/EtOH-induced coleoptile tissue microsomes.
[b] Means ± SD of triasulfuron hydroxylase activity from three different coleoptile microsome preparations.

TABLE 7
Effect of Cytochrome P450 Inhibitors on Wheat Microsomal Triasulfuron Hydroxylase Activity[a]

Inhibitor	Concentration (μM)	% Inhibition[b]
Cytochrome *c*	0.5	76 ± 6 (36)[c]
Piperonyl butoxide	100	77 ± 2 (60)
Tetcyclacis	100	78 ± 4 —
Paclobutrazol	100	36 ± 0 (18)

[a] NA/EtOH-induced coleoptile tissue microsomes.
[b] Means ± SD from two different coleoptile microsome preparations.
[c] Results of previous studies (12) with NA-induced Sephadex G-25 microsomes from etiolated shoot tissues in parentheses.

sulfuron binding spectra with the cytochrome P450 in coleoptile microsome preparations were also observed (Fig. 1B). The weak binding spectra with triasulfuron compared to the strong binding spectra with tetcyclacis also suggested that the cytochrome P450 isoform responsible for triasulfuron hydroxylation may represent only a small fraction of the total cytochrome P450 present in the induced microsomal preparations.

Polyclonal antibodies prepared against a partially purified wheat microsomal NADPH cytochrome c reductase fraction also inhibited both NADPH cytochrome c reductase activity and triasulfuron hydroxylase activity of NA/EtOH-induced coleoptile microsomes (Table 8).

Inhibition of triasulfuron hydroxylase activity was proportional to antibody concentration. In contrast, NADPH cytochrome c reductase inhibition was not proportional to antibody concentration. This may suggest that the polyclonal antibodies raised against the Superose 12 middle fraction of NADPH cytochrome c reductase activity were effective inhibitors of a NADPH cytochrome c reductase isoform associated with triasulfuron hydroxylase activity, but were less effective inhibitors of other possible NADPH cytochrome c reductase isoforms present in induced wheat coleoptile microsomal preparations.

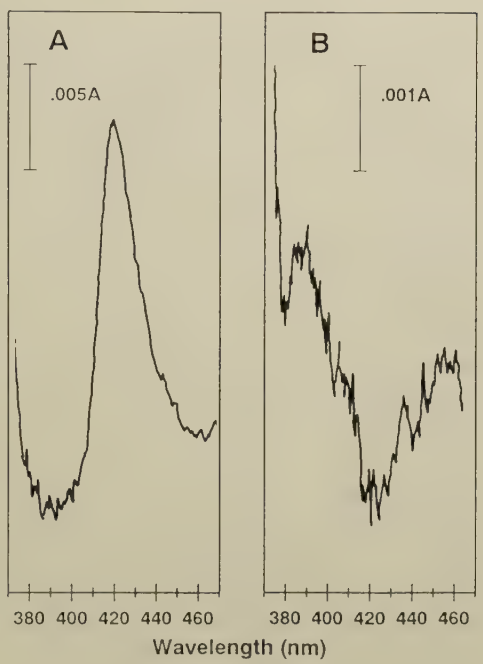

FIG. 1. *Induced microsomal cytochrome P450 binding spectra. (A) Type II difference spectrum with tetcyclacis: Sample cuvettes contained 0.8 mg microsomal protein (482 pmol cytochrome P450 mg^{-1} protein), 100 μM tetcyclacis (10 μl DMSO), and 0.1 M potassium phosphate buffer (pH 7.5) in a final volume of 1.0 ml.* (B) Type I difference spectrum with triasulfuron: Sample cuvette contained 1.5 mg microsomal protein (*466 pmol cytochrome P450 mg^{-1} protein), 60 μM triasulfuron (10 μl DMSO), with 2.5 mM DTT and 0.1 M potassium phosphate buffer (pH 7.5) in final volume of 1.0 ml. Reference cuvettes contained DMSO (10 μl) without added tetcyclacis or triasulfuron. NA/EtOH-induced coleoptile tissue microsomal preparations.*

Light reversibility of CO inhibition and studies with a number of specific inhibitors of cytochrome P450 or associated NADPH cytochrome P450 reductase activity provide further evidence for the direct involvement of a cytochrome P450 in triasulfuron hydroxylation by wheat microsomes.

Reconstitution of triasulfuron hydroxylase activity. Major problems in the isolation and purification of specific cytochrome plant cytochrome P450s are that initial concentrations of constitutive cytochrome P450 are very low and that membrane solubilization with detergents often results in

TABLE 8

Inhibition of Microsomal Enzyme Activities by Rabbit Polyclonal Antibodies Raised against NADPH Cytochrome c Reductase from Etiolated Wheat Shoots

Treatment[a]	NADPH cytochrome c reductase (% inhibition)	Triasulfuron hydroxylase (pmol min^{-1} mg^{-1} protein)[b]	Triasulfuron hydroxylase (% inhibition)
Control	—	1226 ± 117	—
Preimmune IgG (55 μg)	14	1274 ± 9	0
Preimmune IgG (110 μg)	9	1269 ± 59	0
Anti-Reductase (50 μg)	48	803 ± 61	35
Anti-Reductase (100 μg)	59	395 ± 84	68

[a] Microsomes from NA/EtOH-induced coleoptile tissues incubated with purified antibodies for 1 hr at 4°C prior to enzyme assays.

[b] Duplicate assays ± SD with separate microsome preparations.

substantial losses of enzymatic activity. Without reconstituted enzymatic activity, it is very difficult to isolate and detect cytochrome P450 isoforms that are responsible for specific metabolic reactions. Consequently, a reconstituted cytochrome P450-dependent monooxygenase system from wheat that hydroxylates triasulfuron was developed.

While a partial purification of cytochrome P450 resulted in a somewhat higher specific cytochrome P450 content than the original microsomes, large losses of cytochrome P450 were incurred. Appreciable increases in the level of inactive cytochrome P420 were not observed, but cytochrome P450 losses of 60% or greater during purification were often observed. Possible denaturation during concentration steps in the presence of KCl may have been a factor in the poor cytochrome P450 recoveries. Rather than concentrating fractions eluted from the Protein-Pak DEAE column and freezing them directly, a better procedure may be to remove excess detergent and salt before the fractions were concentrated, frozen, and stored at −70°C. This approach was not tested.

The NADPH cytochrome c reductase fraction used in the reconstitution experiments was purified by affinity chromatography on a 2′,5′ ADP-Sepharose 4B column as described. A purification of 340- to 750-fold over the starting microsomal NADPH cytochrome c reductase specific activity was obtained and these enzyme preparations were used in the reconstitution assays (data not shown).

When purified NADPH cytochrome c reductase and cytochrome P450 fractions were chromatographed separately on an hydroxyapatite column to remove detergent and then incubated together in the reaction mixture with phospholipid and [^{14}C]triasulfuron (System I, Table 9), reconstituted triasulfuron hydroxylase activity was approximately threefold greater (3.1 pmol min^{-1} mg^{-1} protein) than controls without NADPH cytochrome c reductase (1.1 pmol min^{-1} mg^{-1} protein) or cytochrome P450.

In contrast, when purified NADPH cytochrome c reductase and cytochrome P450 fractions were combined, chromatographed together on hydroxyapatite, and then assayed for triasulfuron hydroxylase activity in the presence of phospholipid and [^{14}C]triasulfuron (System II, Table 9), the efficiency of reconstitution was greatly enhanced. Cochromatography on hydroxyapatite appeared to facilitate a more efficient interaction between NADPH cytochrome c reductase and cytochrome P450 in the reconstituted system. Results of a typical as-

TABLE 9
Triasulfuron Hydroxylase Reconstitution[a]

Reaction systems[b]	Cyt. P450 (pmol)	NADPH cytochrome *c* reductase units (nmol min^{-1})	Triasulfuron hydroxylase activity (pmol min^{-1} mg^{-1} protein)
I			
Cyt. P450 + reductase + phospholipid	240	560	3.1 [1.0][c]
Cyt. P450 + phospholipid	240	560	1.1 [0.4]
Cyt. P450 + reductase	240	560	2.8 [0.9]
Phospholipid + reductase	—	560	[d]
II			
Cyt. P450 + reductase + phospholipid	270	625	31.8 [10.3]
Cyt. P450 + reductase + phospholipid	135	312	49.1 [15.8]
Cyt. P450 + reductase + phospholipid	67	156	62.7 [20.2]

[a] Reaction mixtures contained indicated amounts of cytochrome P450 and/or NADPH cytochrome c reductase, 10 μg of phospholipid (dilauroylphosphatidylcholine), and 4 nmol of [^{14}C]triasulfuron in a final volume of 250 μl (I) or 325 μl (II).

[b] Isolated cytochrome P450 and NADPH cytochrome c reductase fractions were chromatographed separately on hydroxyapatite (I) or combined and chromatographed together on hydroxyapatite (II) prior to addition to the reaction mixture.

[c] Fold increase in brackets.

[d] The NADPH cytochrome c reductase fraction contained a trace of cytochrome P450. Hydroxylation rates for phospholipid + reductase and Cyt. P450 + phospholipid were comparable.

say for reconstituted (System II) triasulfuron hydroxylase activity are shown in Fig. 2.

When purified NADPH cytochrome c reductase and cytochrome P450 fractions were combined and the detergent was removed by chromatography on hydroxyapatite, triasulfuron hydroxylase activity var-

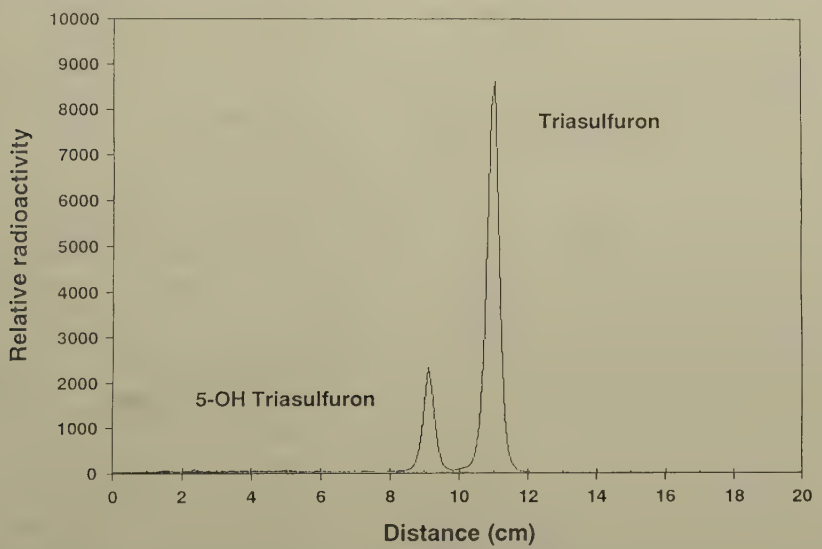

FIG. 2. *Formation of 5-OH triasulfuron by reconstituted triasulfuron hydroxylase (system II). Reaction contained 415 pmol of cytochrome P450, 960 units (nmol min^{-1}) of NADPH cytochrome c reductase, 10 μg of dilauroylphosphatidylcholine, and 4 nmol of [^{14}C]triasulfuron in a final volume of 300 μl. TLC solvent system was benzene:acetone:formic acid (30:10:1, v/v).*

ied from 31.8 to 62.7 pmol min^{-1} mg^{-1} protein depending on cytochrome P450 concentration (System II, Table 9). The highest specific activity (62.7 pmol min^{-1} mg^{-1} protein) was about 6% of the microsomal rate of 1038 pmol min^{-1} mg^{-1} protein (Table 4). No attempt was made to optimize the reaction conditions in terms of cytochrome P450/NADPH cytochrome c reductase ratios, phospholipid concentration, ionic strength, or pH. However, preliminary studies with added FAD, FMN, and BSA in the reaction mixture (9) did not increase reconstituted triasulfuron hydroxylase activity.

Even though the initial reaction rates with a reconstituted enzyme system were low compared to the microsomal activity, they may be sufficient, at least as a starting point, to permit the development of improved reaction conditions needed for the isolation and further characterization of an induced cytochrome P450 isoform responsible for triasulfuron hydroxylation in wheat. Hopefully, the partial purification and demonstrated reconstitution of an inducible microsomal cytochrome P450-dependent enzyme responsible for the metabolic detoxification and selectivity of triasulfuron in wheat will provide the basis for an eventual identification of the gene(s) that regulate triasulfuron activity and tolerance in plants.

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The Significance of Pharmacokinetics and Metabolism to the Biological Activity of RH-5992 (Tebufenozide) in *Spodoptera exempta, Spodoptera exigua,* and *Leptinotarsa decemlineata*

GUY SMAGGHE AND DANNY DEGHEELE

Laboratory of Agrozoology, Faculty of Agricultural and Applied Biological Sciences, University Gent, Coupure Links 653, B-9000 Gent, Belgium

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Toxicity assays indicated that RH-5992 (tebufenozide), a novel synthetic nonsteroidal ecdysteroid agonist, induces a premature, abnormal, and lethal molt in last instar larvae of the African armyworm, *Spodoptera exempta* (Walker), and the beet armyworm, *Spodoptera exigua* (Hübner), by means of an oral application. However, toxicity values differed markedly between the two species. The LC_{50} value for *S. exempta* was 0.034 mg/liter compared to 2.5 mg/liter for *S. exigua*. On the other hand, RH-5992 had no significant effect on larval growth, development, or adult formation of last instar larvae of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), at concentrations up to 50 mg/liter. However, at 100 mg/liter, neurotoxic effects were observed in some cases. To explain the different toxicity to both *Spodoptera* spp. and *L. decemlineata,* pharmacokinetics and metabolic detoxification of ^{14}C-labeled RH-5992 have been determined. In all three insect species excretion of the absorbed compound was high, with a 50% excretion time of 2–3 hr after oral uptake. Data on distribution and metabolism of [^{14}C]RH-5992 suggest that the observed different biological activity in both *Spodoptera* spp. and *L. decemlineata* cannot be attributed to pharmacokinetic and metabolic differences. © 1994 Academic Press, Inc.

INTRODUCTION

RH-5992[1] (tebufenozide) is a novel synthetic nonsteroidal ecdysteroid agonist, succeeding the prototype RH-5849 which binds to the EcR competitively with ecdysteroids, resulting in a toxicological effect with a significant mode of action (1, 2). Caterpillars treated with RH-5849 exhibited symptoms of a prematurely induced lethal larval molt within 24 hr after treatment (2–10). For RH-5992, a similar activity is suggested, but with a much higher potency and selectivity toward lepidopterous species than RH-5849 (11–13). On the other hand, these compounds showed an inconsistent activity or no activity on insects of other orders, e.g., Coleoptera, Diptera, Heteroptera, and Orthoptera (3, 8, 10, 13, 15).

The present study evaluates biological activity, pharmacokinetics, and metabolism of RH-5992 in three important pest insects, i.e., the African armyworm, *Spodoptera exempta* (Walker), the beet armyworm, *Spodoptera exigua* (Hübner), and the Colorado potato beetle, *Leptinostarsa decemlineata* (Say). This research is aimed at providing evidence for the novel mode of action of RH-5992 and at obtaining a better understanding of its relatively high selectivity for Lepidoptera compared to the prototype analog RH-5849. In addition, this study evaluates pharmacokinetic and metabolic aspects, aiming specifically at elucidating differential toxicity of ecdysteroid agonists on various insect species.

[1] Abbreviation used: ANOVA, analysis of variance; EcR, ecdysteroid receptor; EH, eclosion hormone; IGR, insect growth regulator; LC_{25}, LC_{50}, and LC_{95}, concentrations required to affect or kill 25, 50, and 95% of the treated larvae, respectively; LC-p line, log (concentration)-probit line; LSD, least significant difference; RH-5992, 3,5-dimethylbenzoic acid 1-1(1,1-dimethylethyl)-2(4-ethylbenzoyl)hydrazide; SD, standard deviation; 95% CL, 95% confidence limits.

MATERIALS AND METHODS

Chemicals

Technical RH-5992 was obtained from Rohm and Haas Company (Spring House,

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PA). The radiolabeled isotope used was [^{14}C]RH-5992, with a specific activity of 23.06 mCi/g, labeled in the t-butyl group and synthesized at Rohm and Haas Company. The labeled compound was diluted in methanol. The purity of both compounds was verified by TLC, following the procedure described under *In Vivo* Metabolism. All solvents were of analytical grade.

Insects

All insects were reared under standard laboratory conditions. Larvae of *S. exempta* were cultured on freshly cut maize seedlings and kept at 28°C, 60% RH, and a 16:8 (L:D) photoperiod (16). *S. exigua* was fed on fresh castor bean leaves, *Ricinus communis* L. and reared at 23°C, 70% RH, and a 15:9 (L:D) photoperiod (17). All stages of *L. decemlineata* were reared on freshly cut potato foliage, *Solanum tuberosum* L. "Bintje" at 23°C, 65% RH, and a 16:8 (L:D) photoperiod (18).

Toxicity and Biological Activity Assay

Different concentrations of RH-5992 were prepared in water containing 0.02% Triton X-100. Freshly cut leaves were dipped in the solution for 10 sec, dried for 40 min at room temperature in a fume cupboard, and provided to the larvae *ad libitum*. Leaves treated with water and 0.02% Triton X-100 were provided to the control larvae. For each dose, three to four replicates of 10 newly ecdysed last instar larvae (0–2 hr), kept in an air-vented plastic petri dish (14 × 2 cm), were used. At the end of the last instar, some wood shavings (*Spodoptera* spp.) or moist vermiculite (*L. decemlineata*) were placed in the petri dish to allow pupation. Mortality counts were done after 6 days for both *Spodoptera* and 13 days for *Leptinotarsa*. At each assessment, larvae were classified as affected when showing abnormal growth or behavior compared to controls. Mortality percentages included both dead and affected larvae. The toxicity data were corrected for untreated mortality and subjected to probit analysis using POLO-PC procedure (19).

Treated and control larvae were examined at various intervals for their biological parameters such as weight gain, feeding, molting, and pupation. Data on larval weight were subjected to one-way ANOVA and means were separated by LSD multiple range test ($P \leq 0.05$) (20).

For internal abnormalities, control and treated larvae were collected and fixed in 4% formaldehyde for 12 hr. Subsequently, they were rinsed in distilled water, dehydrated by passage through ethanol and xylol, and then embedded in paraplast. Cross sections were made with a Historange microtome prior to examination with a light microscope.

Treatment of Last Instar Larvae with Radiolabeled [^{14}C]RH-5992

Newly ecdysed last instar larvae (0–2 hr) were reared individually and starved for 6 hr before use in order to induce a higher feeding rate. Two microliters of [^{14}C]RH-5992 was dispersed on a fresh maize, castor bean, or potato leaf disc (7 mm diameter). After the solvent was evaporated, one treated disc was offered to *S. exempta, S. exigua,* or *L. decemlineata,* respectively. The average amount of radioactivity on the leaf disc was 28,972 ± 2323, 29,194 ± 2544, or 31,074 ± 1789 dpm, respectively. After the disc was eaten completely (~after 15 min), untreated freshly cut leaves were provided. Larvae which had not entirely consumed the disc were removed. For each treatment, larvae and their excrements were collected separately at various time intervals and kept at −20°C until analysis.

Determination of Radiolabeled Residues in the Larval Body and the Excrements

Three to four replicates of two complete larvae as well as their excrements were combusted separately in a Packard Biological Material Oxidizer Model 306. The combusted gases (+[^{14}C]dioxide) were collected with 8 ml "Carbosorb I" (Packard)

and 10 ml "Permafluor E+" (Packard). The radioactivity was determined by a Kontron liquid scintillation counter and quenching was automatically checked with an external standard ratio. All results were expressed in terms of percentage of the applied amount of [^{14}C]RH-5992 (in dpm).

Determination of Radiolabeled Residues Retained in the Cuticle, the Food Canal, and the Hemolymph

The distribution of radiolabeled residues of [^{14}C]RH-5992 was determined by dissecting three to four replicates of two larvae on a Whatman filter paper No. 3 to obtain the cuticle (includes carcass + muscles + peritoneal fat body + head), the food canal (+content + visceral fat body + malpighian tubes), and the hemolymph (the amount absorbed by the filter paper). Combustion and determination of the radiolabeled residues and statistical analysis of the results were carried out as mentioned above.

In Vivo Metabolism

Three to four replicates of five larvae of each insect species as well as their excrements were collected separately, frozen, and lyophilized for 24 hr. RH-5992 and possible metabolites were extracted by homogenizing the freeze-dried samples with an Heidolph electrohomogenizer for 10 sec in 1 ml of ethyl acetate at 0°C. Subsequently, 1 ml of acetonitrile and 1 ml of isopropanol were added. After each addition of organic solvent, the homogenate was vigorously vortexed for 1 min and precipitated by standing at room temperature. The combined supernatant was evaporated under vacuum to yield a volume of about 50–100 µl, and the extracts were then spotted on TLC aluminum sheets covered with silica gel 60F_{254} (Merck 5554). The chromatograms were developed in dichloromethane:acetonitrile:acetic acid (67:33:1). The radioactivity of the spots of original [^{14}C]-RH-5992 and of the total amount of metabolites was determined by using 10 ml "Lipoluma" (Lumac LSC) and a liquid scintillation spectrophotometer (Kontron) with an automatic quench correction to calculate dpm values. The average percentage of recovery was 75.8 ± 7.9.

RESULTS

Toxicity and Biological Activity Assay

RH-5992 was about 60 to 80 times more toxic to last instar larvae of *S. exempta* compared to *S. exigua* (Table 1). At concentrations of ≥0.02 mg/liter in *S. exempta* and ≥1 mg/liter in *S. exigua* symptoms of a prematurely induced and lethal molt were visible. Within 12–24 hr after application, a new head capsule was formed, revealing a double head capsule; moreover, underneath the old cuticle a fragile and (often) nonsclerotized new head capsule was ob-

TABLE 1

Toxicity of RH-5992 to Last Instar Larvae of S. exempta, S. exigua, and L. decemlineata, Expressed by LC Values and Slopes

Insect species	LC_{25} (95% CL)	LC_{50} (95% CL)	LC_{95} (95% CL)	Slope LC-p line
S. exempta	0.019 (0.014–0.024)	0.034 (0.028–0.042)	0.139 (0.102–0.222)	2.71
S. exigua	1.5 (0.9–2.0)	2.5 (1.8–3.4)	8.8 (6.0–14.8)	3.00
L. decemlineata	—[a]	—[a]	—[a]	—

Note. 95% CL in parentheses.

[a] No significant effects were observed at concentrations up to 50 mg/liter in last instars of *L. decemlineata*. However, at 100 mg/liter, signs of a neurotoxic effect were noted in some cases.

served. Figure 1 exemplifies the formation of a double cuticle in the thorax of a treated last instar of *S. exempta* and *S. exigua*. Likewise, larval weight gain of treatments was significantly suppressed ($P \leq 0.05$) and in some cases loss of hemolymph and extrusion of the hindgut were observed. Most larvae died in their old cuticle shortly afterward. In some cases the old cuticle was partially shed, with remnants remaining on the new cuticle. Several last instars of *S. exempta* and *S. exigua*, treated with concentrations ranging from 0.01 to 0.05 mg/liter and from 1 to 10 mg/liter, respectively, and which did not appear to be affected, later showed an abnormal and lethal pupation. They were unable to ecdyse successfully or to build up a normal pupal cuticle, resulting frequently in larva–pupa intermediates. At ≤0.001 mg/liter and ≤0.05 mg/liter, RH-5992 had no effect on larval growth and development of *S. exempta* and *S. exigua*, respectively. On the other hand, RH-5992 at a concentration up to 50 mg/liter, had no effect on molting or behavior of last instar *L. decemlineata* larvae, resulting in cuticle build-up (Fig. 1), weight gain ($P > 0.05$), and adult formation similar to that in the controls. However, at 100 mg/liter, neurotoxic symptoms were visible in some cases. Some larvae showed tremors, followed by paralysis shortly afterward, while others survived these effects.

Determination of Radiolabeled Residues Retained in the Larval Body and the Excrements

One hour after intake, 72 and 93% of the applied amount of [^{14}C]RH-5992 were retained in the body of *S. exempta* and *L. decemlineata*, respectively. Further on, these amounts of ^{14}C radioactivity decreased rapidly. Figure 2 points out that the pattern of excretion appeared to be approximately a single exponential time course in both species, while in *S. exigua*, the reduction of radiolabeled residues was less pronounced, resulting in a respective decline to 86 and 49% at 2 and 3 hr after uptake. Further on, the amount of radioactivity decreased to 28 and 15% between 6 and 12 hr after uptake (Fig. 2).

Likewise, excretion of the absorbed compound occurred rapidly after being accumulated relatively fast in the body tissues. In all three species, 50% of the applied amount was recovered in the excrements 2–3 hr after uptake. Although the half-life values were almost equal, a difference was observed in the way the radiolabeled residues were later excreted. The excretion rates after 50% of excretion were slower in *S. exigua* compared to *S. exempta* and *L. decemlineata*. In this way, 70–80% of the applied amount was already excreted 3–4 hr after intake in *S. exempta* and *L. decemlineata*, while in *S. exigua*, a similar percentage was only obtained 12 hr after uptake (Fig. 2).

Determination of Radiolabeled Residues Retained in the Cuticle, the Food Canal, and the Hemolymph

Following uptake of [^{14}C]RH-5992, our data confirmed a fast absorption from the food canal into the hemolymph and cuticle in the three species. In *S. exempta*, a total of 31 and 24% of the applied dose remained 1 hr after oral uptake in its hemolymph and cuticle; in *S. exigua* this was 20 and 24% and in *L. decemlineata* 21 and 15%, respectively (Fig. 3). Further on, these amounts decreased in *S. exempta* and *L. decemlineata*, yielding a constant basal level of about 1% at 12 and 3 hr after uptake, respectively. While in the hemolymph and cuticle of *S. exigua*, the label reached similar low levels only at 24–48 hr after uptake.

The pattern of radioactivity in the food canal of *S. exempta* was similar to that observed in its hemolymph. In *S. exigua*, very high amounts were noted during the first hours. Further on, they stabilized to reach a level of 10–15% between 3 and 4 hr, and from 12 hr onward the amount of label remained at about 2%. In the food canal of *L. decemlineata*, the percentage of radioactivity reached very high levels during the first

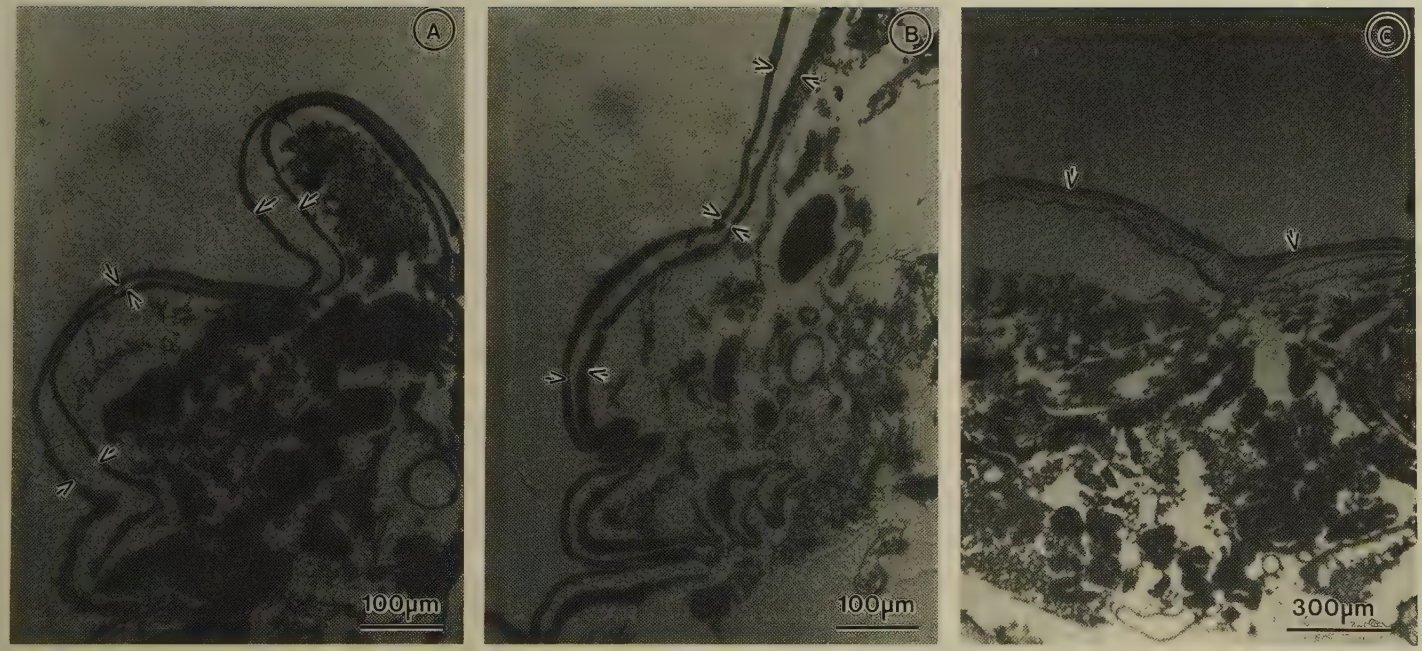

FIG. 1. *Cross section through the thorax of a last instar S. exempta (A) and S. exigua larvae (B) fed for 24 hr on leaves treated with 0.05 and 3 mg/liter RH-5992, respectively; L. decemlineata (C) for 48 hr with 50 mg/liter. Arrows indicate cuticle formation.*

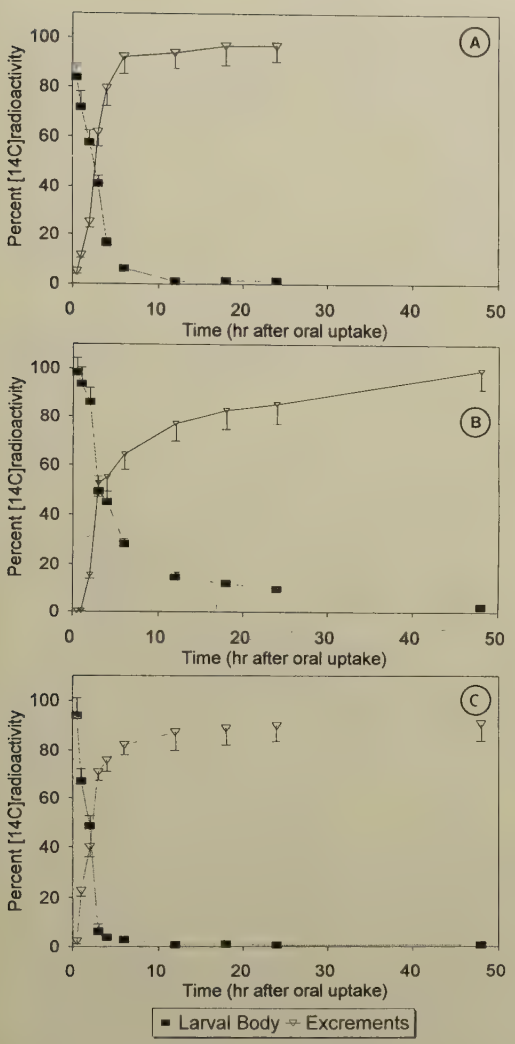

FIG. 2. *Retention of radiolabeled residues of [^{14}C]RH-5992 in the insect body and the excrements of last instar larvae of S. exempta (A), S. exigua (B), and L. decemlineata (C). Data are mean values ± SD expressed as percentages of the mean amount of [^{14}C]-RH-5992 applied per specimen (in dpm).*

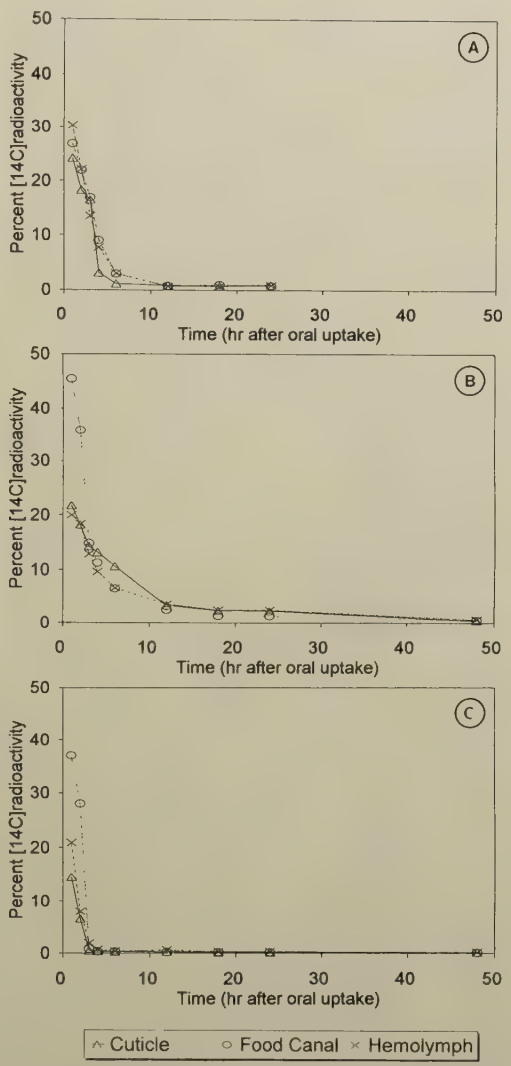

FIG. 3. *Distribution of radiolabeled residues of [^{14}C]RH-5992 in the cuticle, the food canal, and the hemolymph of last instar larvae of S. exempta (A), S. exigua (B), and L. decemlineata (C). Data are mean values expressed as percentages of the mean amount of [^{14}C]RH-5992 applied per specimen (in dpm). SD values for the three to four determinations were <10%.*

hours after uptake, afterward being very rapidly excreted, resulting in a basal level of about 1% already 3 hr after uptake (Fig. 3).

In Vivo Metabolism

Our data show that RH-5992 was excreted relatively fast out of the larval body of the three insect species as original compound, after having rapidly been accumulated in their body tissues. In the larval body of *S. exempta, S. exigua,* and *L. decemlineata,* 40, 49, and 52%, respectively, of the radiolabeled residues were present as parent ^{14}C-labeled RH-5992 at 2 hr after uptake. Two hours later, these percentages had decreased to 8, 17, and 11%, respec-

tively. In *S. exempta,* the amount of parent compound had stabilized to 0.5% at 12 hr after uptake. Three percent was recovered as original RH-5992 at 24 hr in *S. exigua,* and 24 hr later this was about 1%. In the larval body of *L. decemlineata,* metabolism was of minor importance. However, excretion of parent compound occurred very fast, resulting in a constant level of about 1% already 6 hr after uptake (Fig. 4).

In the excrements of *S. exempta, S. exigua,* and *L. decemlineata,* the amount of parent [^{14}C]RH-5992 increased rapidly and remained relatively high with the increase in time. Two hours after uptake, 4, 1.5, and 6% of the applied amount was recovered as parent compound in the excrements of *S. exempta, S. exigua,* and *L. decemlineata,* respectively. Later, these percentages increased, reaching a level of 34 and 41% in *S. exempta* at 6 and 12 hr after uptake; in *L. decemlineata* a level of 34 and 40% was

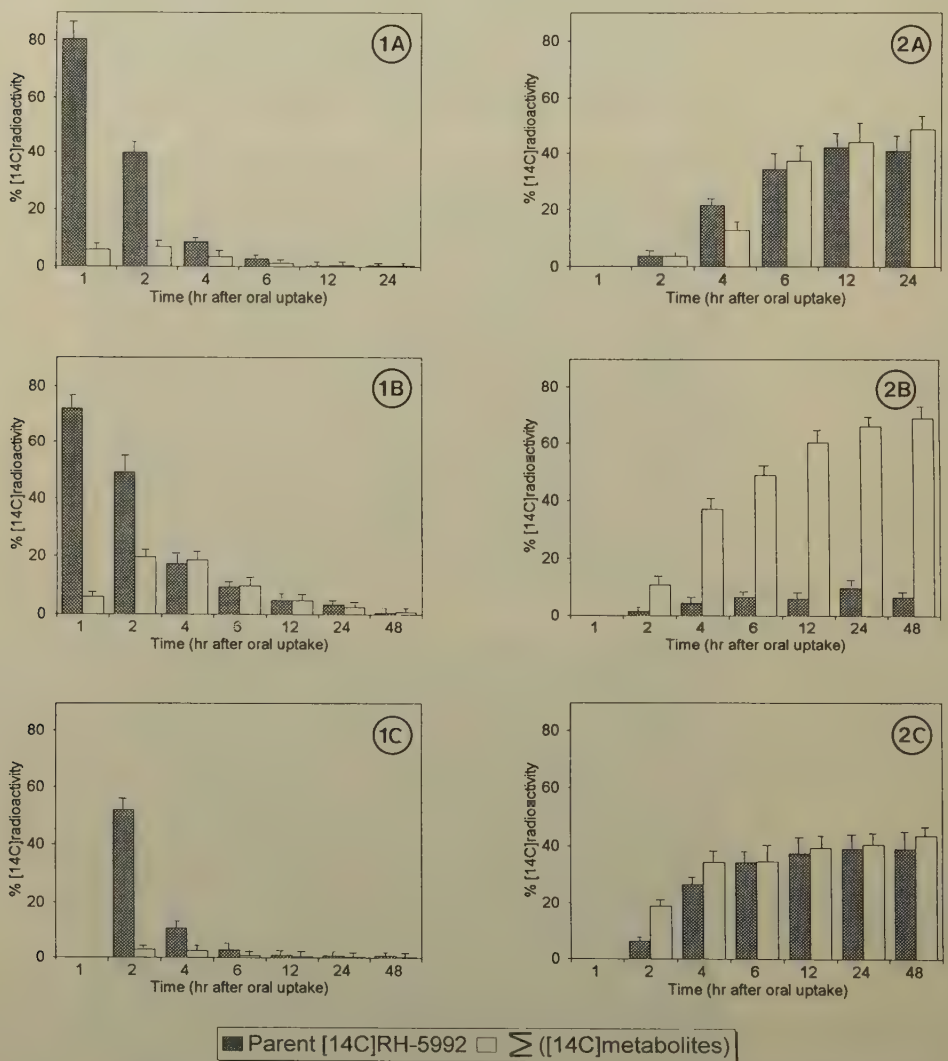

FIG. 4. *Metabolism of* [^{14}C]*RH-5992 in the insect body* (*1*) *and excrements* (*2*) *of last instar larvae of S. exempta* (*A*), *S. exigua* (*B*), *and L. decemlineata* (*C*). *Data are mean values ± SD of original* [^{14}C]*RH-5992 and the total amount of labeled metabolites, both expressed as percentages of the mean amount of* [^{14}C]*RH-5992 applied per specimen* (*in dpm*).

found at 6 and 12 hr after uptake. On the other hand, the quantity of parent RH-5992 in the excrements of *S. exigua* remained fairly constant at 7% from 6 hr after uptake onward (Fig. 4).

Following oral uptake of [^{14}C]RH-5992, the amount of metabolites recovered in the larval body was relatively low (Fig. 4). In *S. exempta* and *L. decemlineata,* it never reached a total amount of 10%, while in *S. exigua,* 20% of the applied dose was present as metabolites at 2 and 4 hr after uptake. In the excrements, higher levels of metabolites were extracted. In *S. exempta* and *L. decemlineata,* the amount of metabolites were extracted. In *S. exempta* and *L. decemlineata,* the amount of metabolites reached a level of 40% of the applied amount of radioactivity around 6 hr after uptake. In the excrements of *S. exigua,* this amount reached this percentage at 4 hr after uptake, and it increased further to 66 and 70% between 24 and 48 hr after uptake (Fig. 4).

DISCUSSION

The results of this study demonstrate that RH-5992 primarily acts by inducing a lethal premature molt in both larval Lepidoptera. It stimulated the epidermal cells to undergo apolysis and to synthesize a new larval cuticle. However, the inability of treated larvae to ecdyse out of the old cuticle may be provoked by an interference with EH production since it is known that this hormone is released as a result of a drop in the ecdysteroid titer after a peak level, prior to ecdysis (21). In this respect, residual amounts of RH-5992 in the insect body may have caused an inhibition of EH release. Similar effects of death during a promoted molt have previously been reported in other catepillars treated with synthetic ecdysteroid agonists or natural ecdysteroids (2–13, 15, 22). Likewise, suppression of weight gain and feeding in treated larvae of *S. exempta* and *S. exigua* may result from an ecdysonergic action of RH-5992. In general, cessation of feeding occurs prior to pupation as a result of ecdysteroid secretion (23).

In addition, the LC_{50} ratio of RH-5992 between last instar larvae of *S. exigua,* and *S. exempta* was 73. Similarly, this ratio of RH-5849 for similar species was 61 (10). When RH-5992 was topically applied to last instars, the ratio of LD_{50} (*S. exigua*)/LD_{50} (*S. exempta*) was 7.5 (13). In another assay, a similar ratio was obtained for RH-5849 (15). Thus, the susceptibility of both species and the toxicity pattern of both compounds showed a constancy, suggesting both compounds act in a relatively similar way in both species.

At doses up to 50 mg/liter RH-5992 had no activity on larval growth and development of last instars of *L. decemlineata.* This agrees with the results of G. R. Carlson (personal communication, 1993, 1994), finding RH-5992 to be 200–3000 times less active on *L. decemlineata* than RH-5849. In this way, it was also found that when applying an extremely high dose of 2500 mg A.I./liter using a 2F formulation of RH-5992, treated last instar larvae of *L. decemlineata* showed signs of a prematurely induced larval molt (own unpublished data). These results agree with those obtained for RH-5849, but at lower concentrations (10).

In *L. decemlineata* only, a neurotoxic effect was noted, resulting in some cases to larval death. In some other treatments treated larvae survived the effect. As suggested for RH-5849, these effects may be provoked by a direct action on K^+ channels and may not involve EcR activation (24). Although we observed no neurotoxic symptoms in treated Lepidoptera, which is consistent with other reports (2–13, 15), neurotoxicity has been reported in caterpillars, only by the injection of very high doses (24). Thus, it can be concluded that ecdysteroid agonists may have different sites of activity; however, many questions remain to be answered. In general, we expect that similar target sites are present in Lepidoptera and Coleoptera.

In this study we have evaluated two pro-

cesses which might be responsible for the large differences in biological activity of RH-5992 on various insect species. Differences in retention and distribution of RH-5992 in the insect body could play an important role in its toxicity similar to other insecticides (25, 26). However, our study revealed that the label was excreted relatively fast before being rapidly accumulated in the body tissues of both Lepidoptera, since we noted a half-life value of 2–3 hr in both species. Our results also confirmed that between 6 and 24 hr after uptake the amount of RH-5992 present in the insect body of *S. exigua* reached a level which was about 5- to 10-fold that of *S. exempta*. Data on the distribution of RH-5992 in the insect body showed a rapid transport of the compound from the food canal into the hemolymph and the cuticle. Labeled residues were, between 6 and 12 hr after uptake, three to five times more prevalent in the cuticle and the hemolymph of *S. exigua* than of *S. exempta*. Moreover, the excretion rate after 50% of excretion was higher in *S. exempta* than in *S. exigua*. Likewise, the radiolabel was recovered to a higher extent in the gut of *S. exigua* than that of *S. exempta* during the first hours after uptake. In this way, higher residue levels of [^{14}C] label were present in *S. exigua*. Thus, these pharmacokinetic results are in contrast with the toxicity values obtained for last instar larvae of *S. exempta* and *S. exigua*.

Metabolism in the insect body is known to be a powerful factor, which frequently leads to a decreased toxicity of the applied insecticide (17, 26–28). Our results confirm that in the larval body the majority of the applied amount was present as original compound, however, to a varying extent in the different species. In contrast to their susceptibility to the compound as expressed by LC_{50} values, about five times more parent RH-5992 was recovered in *S. exigua* compared to *S. exempta* at 6 hr after uptake.

Our results on metabolism also suggest that detoxication of RH-5992 mainly occurred in the insect food canal. In general, the metabolic stability of RH-5992 in *S. exempta* was greater than that in *S. exigua;* however, in *S. exempta* a higher excretion rate after 50% of excretion was noted. Nevertheless, our data confirm the very high metabolic potency of *S. exigua* compared to other *Spodoptera* spp. present in its food canal and previously demonstrated against several chitin synthesis inhibitors (28).

In *L. decemlineata,* metabolism of RH-5992 seemed to be of minor importance. Moreover, our data showed that the pattern of the amounts of original RH-5992 in *Leptinotarsa* was similar to that in *S. exempta*. These amounts were, however, not active in *Leptinotarsa,* although they induced a premature and lethal molt within 12 hr in *S. exempta*.

The current toxicity assays also confirmed that RH-5992 was more active and more selective for lepidopterous larvae than the prototype RH-5849 (10, 13, 15). In addition, when comparing the current findings and those of a previous study dealing with the pharmacokinetics and metabolism of RH-5849 (10), it is suggested that the higher toxicity of RH-5992 for *S. exempta* and *S. exigua* compared to RH-5849 is not a result of a different retention, distribution, or metabolism.

In general, the major information obtained from the present data is that the observed different toxicity of RH-5992 to the three different insect species cannot be explained by pharmacokinetic and metabolic differences. Thus, its selectivity for lepidopterous species cannot be attributed to these factors. In this way, we expect that further studies dealing with EcR structure and their binding affinity for the ecdysteroid agonist ligand molecules should assist in clarifying the different biochemical mode of action and potency of this novel group of compounds. Hence, the latter hypothesis agrees with the concept that structure and biochemical binding properties of EcRs may vary among insect species (29). Further concerning experiments are in prog-

ress in our laboratory. In addition, our results strengthen the potential use of RH-5992 as a selective insecticide to be used in IPM programs for controlling lepidopterous pests. Moreover, its novel chemistry and mode of action suggest that RH-5992 may be a valuable alternative to control sensitive species resistant to other classes of insecticides, including current IGRs.

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NOTICE

The Subject Index for Volume 49 will appear in the November 1994 issue as part of a cumulative index for the year 1994.

INFORMATION FOR AUTHORS

Pesticide Biochemistry and Physiology will publish original research papers in English, dealing with the biochemistry and physiology of insecticides, herbicides, fungicides, acaricides, nematocides, rodenticides and similar compounds, including nonlethal pest control agents such as antifeeding compounds and chemosterilants as well as, for example, the biosynthesis of pheromones, hormones and plant resistance agents. Topics of interest include the effects of these agents on the biochemistry and physiology of both target and non-target organisms, and the biochemical transformations of the agents themselves. The mechanisms of resistance, and interactions such as synergism and antagonism, are also suitable topics.

It is not intended to publish papers devoid of biochemical or physiological content, e.g., reports of toxicity testing, or chemical synthesis, or analysis of residues (except when used as a tool for elucidation of metabolic pathways), or of the efficacy of pesticide treatments. Nevertheless, suitability is based on the purpose or rationale of the study, and not only upon the technique used.

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3. P. Thomas, R. S. Carr, and J. M. Neff, Biochemical stress responses of mullet (*Mugel ephalus*) and polychaete worms (*Neanthes virens*) to pentochlorophenol, *in* "Biochemical Monitoring of Marine Pollutants" (F. J. Vernberg, A. Calabrese, F. Thurberg, and W. B. Vernberg, Eds.), pp. 73–103, Academic Press, New York, 1981.

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